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A MICROMANIPULATOR FOR THE ISOLATION OF BACTERIA AND THE DISSECTION OF CELLS

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I have recently described (Chambers, 1922, b) an apparatus for the manipulation of micro needles and micro pipettes under the highest magnifications of the microscope. This apparatus is an improvement on Barber's Pipette Holder (Barber, 1914) because of its simpler construction and the greater accuracy with which one can control its movements. An additional advantage consists in the existence of certain devices for bringing the pipette or needle, quickly into position before starting actual operation.

The working principle of the apparatus (which is being patented) is illustrated in figure 1. It consists in the use of bars of rigid metal connected at their ends to form a Z like figure by resilient metal acting as spring hinges. The bars are forced apart by screws and return when the screws are reversed. By these means arc movements are imparted to the tip of a pipette which is attached to one of the bars. As the radius of each arc is about two and a half inches, the fine movements imparted to the tip of the pipette are practically in straight lines because the excursion never exceeds one millimeter.

The instrument can be used by itself for one needle or pipette, or with a companion apparatus when two needles, or a needle and a pipette are to be used simultaneously. When a pair is used, one is a left handed and the other a right handed apparatus, both being clamped to the front of the microscope stage. For the isolation of bacteria, one instrument is sufficient. It may be clamped on the left side of the microscope stage, figure 2, so that the pipette projects into the moist chamber from the

left. The tip of the needle or pipette is bent up so as to project from below into a drop suspended from the coverslip which roofs the chamber. The cells to be operated upon lie in the hanging drop. When a cell is to be dissected or injected it tends to retain its position on account of the shallowness of the drop and the inertia of the cell. However, it is more satisfactory to use two instruments, one with a needle for holding the cell or tissue, and the other with a needle or pipette for the actual operation.

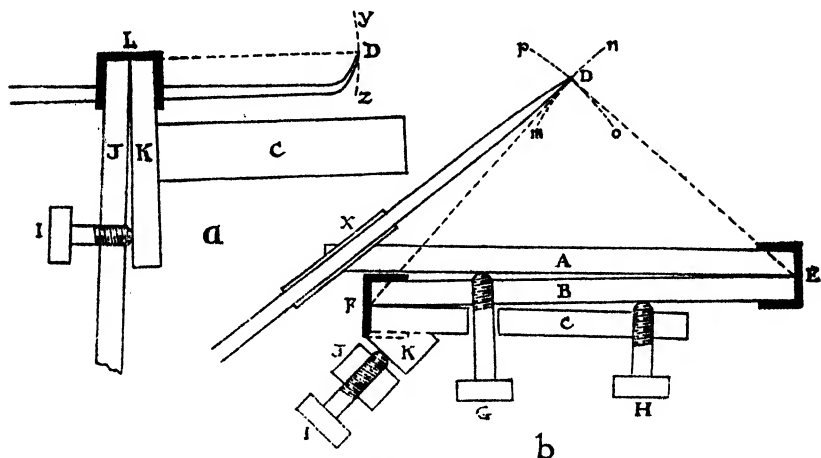


FIG. 1. DIAGRAM SHOWING WORKING PRINCIPLE OF MICROMANIPULATOR

(a) Side view. Screw *I* in stationary pillar *J* pushes against *K*, and causes needle tip *D* to move through vertical arc *y-z*.

(b) Surface view. Screws *G* and *H* move the needle tip through horizontal arcs *m-n* and *o-p*.

For dissecting purposes, the glass needles may be curved or straight and with obtusely or gently tapering tips. They can be made fine enough to puncture red blood corpuscles and to tear up leucocytes.

For injecting and for withdrawing materials from a living cell, the micro pipettes are made with apertures varying from two to less than half a micron in diameter. I have recently described (Chambers, 1922, a) an effective and easily made apparatus for exerting the necessary pressure to drive materials through

such small pipettes, and at the same time to control, with considerable accuracy, the amount to be injected or withdrawn.

For isolating bacteria, much coarser pipettes are used, which can be blown into by the mouth through a length of rubber tubing, figure 2. At my suggestion, Dr. Kahn has kindly pub-

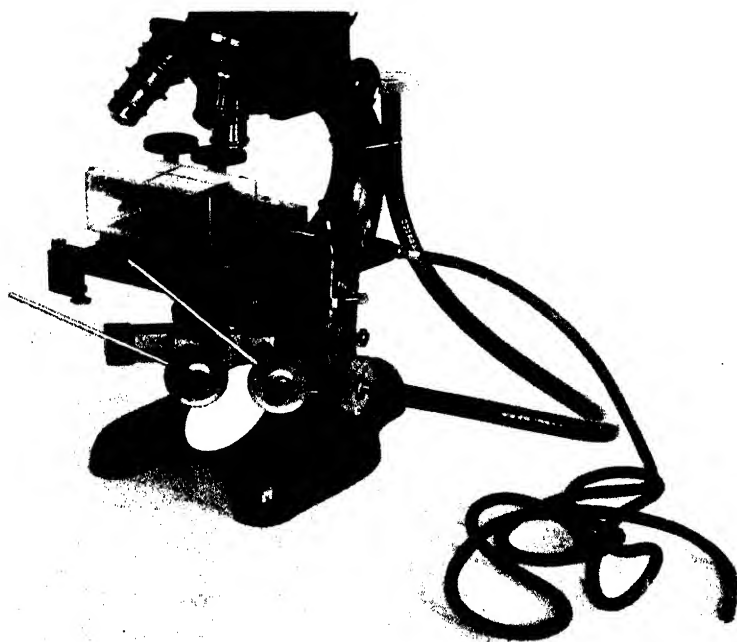


FIG. 2. MICROMANIPULATOR MOUNTED ON LEFT SIDE OF MICROSCOPE FOR ISOLATING BACTERIA

Note Barber's moist chamber with the coverslip marked with cross lines to aid in locating areas. The chamber shown here is higher than necessary. The screw producing the vertical movement is connected with a flexible shaft, which allows its control to be brought into close proximity with the fine adjustment of the microscope.

lished an account (Kahn, 1922) of the procedure, together with a discussion of the application of the micromanipulator to the isolation of bacteria. In brief, the procedure is as follows: A sterile, hollow glass needle is first made. The bept up tip is then inserted into a test tube of a liquid culture of bacteria,

and converted into a pipette by breaking the tip against the wall of the test tube. A small amount of the culture is sucked up, and the filled pipette placed in the micromanipulator attached to the microscope. The tip of the pipette is then brought into the microscopic field and brought close to the coverslip of the

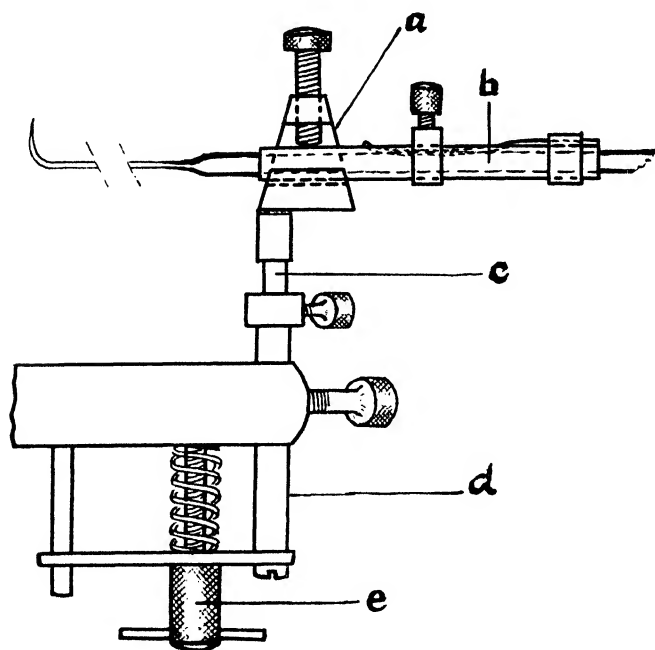


FIG. 3. DETAIL SHOWING DEVICES FOR PRELIMINARY ADJUSTMENTS OF THE PIPETTE

Carrier, *a*, for clamping brass collar, *b*, in which needle or pipette has been inserted. The needle or pipette slides evenly within the collar for the in and out movement. Telescoping pillar, *c*, for lengthening vertical post of carrier. Post, *d*; rotates and serves to move needle tip laterally. Screw, *e*, raises and lowers post, *d*, to move needle tip vertically.

moist chamber by means of the preliminary adjusting devices shown in detail in figure 3. The tip is now further raised by means of the fine adjustment screw until it reaches the under-surface of the coverslip. By alternately raising and lowering the pipette, and by moving the moist chamber with the mechani-

cal stage, a series of hanging droplets¹ are placed on the coverslip. The pipette is then removed from the instrument and discarded. A search is now made for droplets containing only a single organism. Each such droplet is drawn up into a fresh sterile pipette, which is then removed from the instrument and inserted into a tube containing a suitable sterile medium. The contents of the pipette are now expelled by blowing. In this way, one can quickly obtain cultures known to have originated from a single organism.

The micromanipulation technic is not very difficult. The making of the glass needles and pipettes, and the working of the instrument can be quickly mastered.

For the bacteriologist, the isolation method as introduced by Barber, has long proved most successful. With the apparatus described here, it should soon be more widely used.

For the cytologist and cell physiologist, the problem is to find the proper material with which to work. Through micro-operations on certain tissue cells and on such material as Protozoa and marine ova, considerable light has already been thrown upon the nature of living protoplasm.

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¹ Barber uses coverslips smeared with petrolatum to aid in the maintenance of the droplets. The excess, having been washed off with soap and water, the slips are dried with a cloth, and then heated and wiped a second time while still warm. They are sterilized by flaming.

CERTAIN PHASES OF NITROGENOUS METABOLISM IN BACTERIAL CULTURES¹

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The classification of bacteria has been studied from different viewpoints since the researches of Pasteur. With the exception of the three great groups of bacteria, the form of the cell is not a very satisfactory means of differentiation. There is left, then, one method of study that is applicable and that method is the study of the metabolism of the bacteria. The determination of the ability of the bacteria to ferment various carbohydrates has been studied extensively. The study of the nitrogenous metabolism has, however, been confined for the greater part to the identification of such compounds as indol or the determination of the ability of the bacteria to grow in certain nitrogenous media. The paucity of such data is the result of the complication of the subject with an unusual number of factors and, furthermore, of the fact that precise chemical methods have been wanting to give quantitative results.

LITERATURE

Hirschler (1886) was probably the first to observe that the presence of carbohydrate inhibited the production of such nitrogenous decomposition products as indol, phenol and the cresols. Smith (1897) and Peckham (1897) independently came to the conclusion that the production of acid in the medium containing carbohydrate inhibited the formation of indol. This early work

¹ This article represents in part a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Harvard University.

was confirmed later by Glenn (1911) who found that the addition to the medium of more than 0.5 per cent lactic acid inhibited the production of indol. Fischer (1915) concluded, as a result of colorimetric determinations of indol, that neither hydrogen ion content nor total acidity affected its production and that the reason for its absence in sugar media appeared to be the inactivation of the proteolytic enzymes by glucose. Logie (1919-1920) tested for indol colorimetrically with Ehrlich's reagent and found that it decreased in the presence of sugar and increased in its absence. His conclusion that the presence of sugar increased the utilization of indol appears well founded.

Gelatin liquefaction has also been used as an index of the type of nitrogenous metabolism. Glenn (1911) concluded from a series of experiments that the failure of bacteria to liquefy gelatin was due to the acidity developed in media containing carbohydrates. Kendall and Walker (1915) found that a bacteria-free filtrate of a plain broth culture of *P. vulgaris* would liquefy gelatin in the presence of glucose as readily as in its absence. They concluded that the proteolytic enzyme was not produced in cultures in glucose media and that the glucose was fermented first, thus "sparing" the protein of the medium.

The effect of various concentrations of a carbohydrate on the amount and rate of formation of metabolic products has been demonstrated by several workers in this field. Clark and Lubs (1915) showed that an increase in the concentration of glucose, to the limit of the experiment, which was 8 per cent, increased the total amount of gas produced. Bronfenbrenner and Schlesinger (1918) concluded from the results of an extensive series of experiments that the many discrepancies in the results of fermentation studies, which are so apparent in the literature of this subject, have been due largely to the fact that the media have not usually been compared with sufficient attention to the relative proportions of peptone and buffer. At the present time, Kendall and his co-workers and other investigators claim that bacteria prefer carbohydrates and utilize these substances more readily than nitrogenous materials, thus "sparing" the protein of the medium. Kendall and his co-workers have shown that in the

presence of carbohydrate the rate of production of ammonia is usually decreased although the reverse is the case in a few instances.

It is evident from the results quoted above that the presence of carbohydrate in the medium affects the type of the nitrogenous metabolism of the bacteria, so that certain products may not be produced or, if produced, the rate of production may be materially changed. Some investigators have interpreted these changes in the nitrogenous metabolism as indicative of the degree of proteolysis. Kendall and Walker (1915) interpreted ammonia production as an index of proteolysis and concluded that the decreased production of ammonia is proof of the "protein-sparing" action of carbohydrates. Berman and Rettger (1918) used the biuret test of Vernon (1903-1904) as an indicator of proteolysis.

Since amino acids are results of protein hydrolysis, I have assumed that the production of amino nitrogen should give a better index than either ammonia production or the biuret reaction—in other words, that in an actively growing bacterial culture the amino acids are formed as a result of proteolysis. However, I realized that the amount of amino nitrogen of a medium is the difference between the amount produced and the amount utilized by the bacteria and the index is arbitrary to that extent. A similar criticism may be made concerning ammonia production and the biuret reaction as an index of proteolysis. However, the amino nitrogen has the advantage that its production is the result of the direct action of the proteolytic enzymes upon the protein of the medium.

At this point it may be profitable to consider briefly the previous work on amino acid production by bacteria. Before the publication of improved methods, Taylor (1902) made determinations of the amino acids by the laborious method of chemical isolation. He reported that *Bact. coli* did not produce amino acids from casein and concluded that the organism was able to split the protein to albumoses only. With *P. vulgaris*, however, he demonstrated the formation of lysin and histidin. Rosenthal and Patai (1914) increased the virulence of streptococci, staphylococci and *Bact. coli* by means of animal passage, and found that

the more virulent strains gave higher amino nitrogen values when judged by the formol titration method of Sørensen. After the completion of the experimental part of my study Kendall and his co-workers (1922) reported that the amino nitrogen in cultures varied with the organism and also with the presence or absence of sugar. In many tests the amino nitrogen showed a decrease in plain media and an increase in glucose media; although other tests with the same organism showed the reverse. Sears (1916) found that certain bacteria, with the exception of a few strongly proteolytic organisms such as *Ps. pyocyanea* and *B. subtilis*, gave fluctuating values of amino acids from which he concluded that these substances were continuously formed and broken down. Jones (1916) working with *P. vulgaris* in gelatin cultures confirmed the observation of earlier investigators that the gelatin is not liquefied in media containing sugar and came to the conclusion that the proteolytic enzyme was not produced in media containing a fermentable carbohydrate. Waksman (1917) working with *Aspergillus niger* and later (1920) with the Actinomycetes showed that these organisms did not produce as much amino acid nitrogen in glucose media as in plain media. With *Bact. coli* the amino nitrogen was slightly lower in glucose broth than in plain broth. There was an increase in the amino nitrogen in plain broth with *Bact. coli* of 5 mgm. per 100 cc. of the medium. With *Aspergillus niger* there was a decrease in the total amino nitrogen. Robinson and Tartar (1917) working with *B. subtilis*, *B. mycoides* and *Proteus vulgaris* found that the mono-amino and di-amino acids were decreased in blood, egg and peptone media. Wolf and Harris (1916-1917) showed that the total amino acid nitrogen content was decreased by *C. sporogenes* in a medium made by the tryptic digestion of casein. This was confirmed later by Harris (1919-1920) who showed also that the presence of a fermentable carbohydrate increased the total amino nitrogen. Wolf (1918-1919a) obtained the same results with *P. vulgaris*, that is, the presence of glucose increased the amount of amino nitrogen. Wolf and Harris (1918-1919) working with *C. histolyticus* and Wolf working with *Vibrio septique* (1918-1919b) and *C. sporogenes* (1918-1919c) showed that these organisms

increased the total amount of amino nitrogen in cultures. Foster (1921) reported that a decrease in amino nitrogen was associated with an increase in ammonia in cultures of *Streptococcus hemolyticus*. Raistrick and Clark (1921), working with a synthetic medium of tryptophane and tyrosin as the only nitrogen sources, found that the amino nitrogen decreased both in the presence and absence of glycerol. They found that ammonia was not produced in the medium containing glycerol. Their conclusion was that glycerol did not have a "protein-sparing" action.

It is clear from the above brief review of the literature that the studies of the production of amino nitrogen in bacterial cultures have not been very extensive. Furthermore, the results of the various investigators differ very greatly and in many cases the extent of the change in the total amount of the amino nitrogen is surprisingly insignificant.

EXPERIMENTAL

In view of the influence of the medium and other variable factors upon the nitrogenous metabolism of bacteria, the prime importance of having a standard medium, simple in composition and readily reproduced, is at once self evident. The first phase of my work, therefore, consisted in obtaining a suitable medium having the necessary properties and in which the bacteria would grow. After a number of attempts I selected the following: Two per cent Difco peptone made up with mono-potassium and di-sodium phosphates, so that the final hydrogen ion concentration was approximately pH 7.5 and the total phosphorus equivalent to M/20. Glucose, 1 per cent, was added whenever carbohydrate was desired. This was the medium used throughout my work and its method of preparation will be given later in detail. While it was realized that peptone media are not as simple as desirable it was not possible to obtain growth of *C. botulinum* in synthetic media with the amino acids which were available.

The bacteria which were studied were limited to *Bacterium coli*, *Pseudomonas pyocyanea*, *Bacillus subtilis*, *Clostridium botulinum* (type A and type B) and *Clostridium sporogenes*. *Bact.*

coli was selected because it represents a good type which exhibits active fermentative powers. *Ps. pyocyanea* was taken as a type which exhibits weak fermentative power and produces large quantities of ammonia. *B. subtilis*, *C. botulinum* and *C. sporogenes* were selected from the family Bacillaceae which actively decomposes protein. In this group *B. subtilis* was taken as the representative of the aerobes and *C. botulinum* and *C. sporogenes* as representatives of the anaerobes. *C. sporogenes* was selected to compare with the toxin producing organism, *C. botulinum*.

METHODS

Amino nitrogen determination

The nitrogenous metabolism of the bacteria was followed in this study by means of the amino nitrogen and the ammonia nitrogen. At the same time determinations of the glucose content, the hydrogen ion concentration, the amount of phosphorus and the number of bacteria were made.

There were two published methods for the determination of amino acids at the time this study was commenced. The formol titration method of Sørensen (1907-1908) was tried out but was found to be unsatisfactory on account of the color of the culture medium and the presence of phosphates and carbon dioxide. The medium which I used was buffered with phosphates to approximately $M/20$ in terms of phosphorus. Van Slyke's method (1911) using the micro apparatus (1913-1914) was then tried. Control analyses could not be obtained on a sterile 1 per cent peptone solution when this method was used. That the failure to obtain consistent control analyses was not due to the apparatus or reagents was shown by the fact that consistent blanks were obtained by myself and were checked by another worker in the laboratory. The variation in per cent in one particular series of tests was 18.4. Other series gave similar results and occasional tests showed errors even higher than the one indicated.

Dr. Folin very kindly permitted me to use his newly devised method for the determination of amino nitrogen a little over a

year before its publication (1922) and gave me some of the reagent necessary for the determinations. In view of the fact that I have used Folin's method for the determination of amino nitrogen throughout my work a brief discussion of the special applications of the method in bacterial culture media seems pertinent.

In the beginning a grave source of error was detected in the lot of glycocoll which was sold as chemically pure. It was found that the glycocoll solution contained more ammonia nitrogen than amino nitrogen. The water used was ammonia-free and was not the source of the error. It is very important, therefore, to test all lots of glycocoll for ammonia before use as a standard. Dr. Folin very kindly gave me some pure glycocoll with which all standards were prepared that are reported in this paper.

Some time later I made up the reagent of the Folin method for amino nitrogen and experienced difficulty because the standards showed a precipitate after the addition of the thiosulfate and acetic acid solutions. It was thought that there might be some undesirable products left after purification of the reagent and, in order to check this, some of the reagent which Dr. Folin had prepared was obtained from him to compare with the reagent which I had prepared.

It was found with the reagent which I had prepared that there was a certain amount of precipitate formation depending upon the amount of the acetic acid and thiosulfate solutions added. In view of the possibility that this precipitate formation may occur with other workers, it is suggested that the reagent be tested in the nephelometer against a dark field before determinations of amino nitrogen are carried out. In the results which follow, all tests were carried out with 2 cc. of the solution of the amino nitrogen reagent and 2 cc. of the thiosulfate solution. Under these conditions the amount of light which came through the nephelometer was barely perceptible when compared to the other side which had been darkened by holding the hand over the plunger.

Folin method for determination of amino nitrogen. The first problem with this method of the determination of amino nitrogen was to study its constancy with peptone solutions. In order to

do this a flask of 1 per cent Difco peptone was made and sterilized. Samples were removed from day to day and a determination of the amino nitrogen was made. The largest percentage error was 3.5.

Later a 2 per cent Difco peptone solution was used and the results are shown in table 1.

Taking the lowest reading as the correct one, the largest percentage error is 3.0. As shown in table 1, the fact that the results of four out of the five samples were the same suggests that the low determination was faulty. From these findings it is concluded that the method gives consistent results in determining amino nitrogen in peptone solutions.

TABLE 1

Amino nitrogen determinations of a 2 per cent peptone solution. Determinations made on different days

DAY	AMOUNT OF PEPTONE SOLUTION	READING IN MM. STANDARD AT 20 MM.	AMOUNT IN MGM. OF NH_2N
	cc.		
1	0.5	19.0	0.157
1	0.5	19.7	0.152
1	0.5	19.0	0.157
1	0.5	19.0	0.157
2	0.5	19.0	0.157

The next problem was to find whether the addition of a known amount of amino acid nitrogen as the standard glycocoll could be detected. In order to ascertain this a sample of 2 per cent Difco peptone was used as follows: 1 cc. of a 2 to 4 dilution of the peptone gave 0.102 mgm. NH_2N . 1 cc. of a 2 to 4 dilution of the peptone plus 0.05 mgm. of amino nitrogen gave 0.150 mgm. NH_2N . The difference is well within experimental error and shows that the true reading may be obtained after an addition of a standard glycocoll solution. This is illustrative of several similar tests. The same test was applied to culture media after the growth of bacteria and it was found that the presence of metabolic products of the bacteria did not interfere with the test. From these results I concluded that the Folin method for the determination of amino nitrogen was applicable in peptone solutions.

Removal of ammonia. The reagent used in the Folin method for the determination of amino nitrogen reacts with ammonia which, if present, must be removed. It was suggested that the simplest and easiest method for the removal of the ammonia would be with permutit (Folin and Bell, 1917). It was found however, that the permutit removed variable quantities of amino nitrogen and for that reason was inapplicable in this work. The removal of ammonia by the aeration method of Folin (1902-1903) was tested and found to be accurate in the presence of peptone under the conditions of the experiments. Consistent determinations of the amino nitrogen were obtained by using 50 cc. in aeration cylinders when compared with 1 cc. in test tubes. It was further determined that the addition of sodium carbonate and potassium oxalate to the peptone solution in the aeration cylinders did not affect the amino nitrogen.

Ammonia determination. The ammonia content of all solutions was determined by the aeration process of Folin (1902-1903). Tests were carried out to find the speed of the air current and the length of time necessary for complete removal. Much difficulty was experienced with foaming. It was found after trying kerosene and other substances that secondary octyl alcohol (E. K. Co.) was best for this purpose. Tests were made and it was shown that this alcohol did not affect either the readings of the amino nitrogen or the ammonia titration. The procedure finally adopted was the addition of 0.8 to 1 cc. of the alcohol and aeration for two and one half hours.

Phosphorus determination. The buffer content of the medium was judged solely by its phosphorus content. The total phosphorus was determined by titration with uranium acetate.

Hydrogen ion concentration. The determination of the hydrogen ion concentration was made by means of the apparatus described by Bovie (1915). In all solutions the reading was not made until the shifting of the needle had stopped. The only change in the apparatus as originally described was that a galvanometer was used instead of the electrometer.

Glucose determination. The amount of glucose in the medium was determined by the titration method of Folin and Peck (1919),

except when the total amount of sugar was 250 mgm. per 100 cc. or lower, in which case the colorimetric method of Folin and Wu (1920) was used.

Number of bacteria. The aerobic bacteria were plated in plain agar and the counts were made after an incubation period of forty-eight hours at 37°C. An estimate of the approximate number of the anaerobic bacteria was obtained by the method of Breed (1911).

General procedure

The strains of bacteria used were obtained from the sources indicated below and were purified from time to time throughout the experiment. In the case of the aerobes the bacteria were purified by plating on plain agar. The anaerobic bacteria were purified by dilution in glucose agar shake cultures. The cultures were diluted so that only one colony grew in the tube, this procedure being repeated several times with each culture. Tests were made with *C. botulinum* for toxin production. The cultures were obtained from the following sources:

C. botulinum (type A) was isolated by myself from the olives causing the food poisoning outbreak in New York City in 1920.

C. botulinum (type B) was obtained from Mr. P. F. Orr.

C. sporogenes as obtained from Miss Ruth B. Edmondson of the Bureau of Chemistry, U. S. Department of Agriculture, who had received it from Dr. Savage of England.

Bact. coli, *B. subtilis* and *Ps. pyocyanea* were obtained from Dr. Ernst of the Department of Bacteriology, Harvard Medical School.

The medium used was a 2 per cent solution of Difco peptone. The method for making the medium was the same in all cases and was as follows: Thirty-two grams of the peptone were dissolved in about 900 cc. of cold distilled water, 16 cc. of M/2 monopotassium phosphate and 144 cc. of M/2 di-sodium phosphate were added and the whole diluted to 1500 cc. The medium was then placed in a flask and heated for fifteen minutes^a at 15 pounds pressure. The solution was filtered, the loss due to evaporation and absorption by the filter paper was made up and

then it was divided into two lots of 750 cc. each. One lot was diluted with 50 cc. of distilled water and placed in a large bottle holding approximately 900 cc. The second lot was placed in a bottle without further dilution. The bottles were plugged with cotton and a square of paper was tied over the cotton and neck of the bottle. The bottles were then sterilized at 15 pounds pressure for thirty minutes. Eight grams of glucose were dissolved in distilled water, diluted to 50 cc. and sterilized at 15 pounds pressure for fifteen minutes.

The anaerobic bacteria were grown in a partial vacuum obtained by a water pump. Each time the bottles were opened for the purpose of obtaining samples, the vacuum was reestablished and the bottles sealed. Samples were removed on the first, second, fourth and tenth days of growth. The anaerobic bacteria were tested for contamination each time by removing about 1 cc. and plating aerobically. If aerobic growth were shown the bottle was discarded.

On account of the many chemical and bacteriological manipulations which had to be crowded into a short time, it became necessary to systematize the work and the general plan of procedure was as follows: Fifty cubic centimeters of the culture were measured with a graduated pipette and placed in a cylinder for the ammonia aeration. Three grams of potassium oxalate and two grams of sodium carbonate were added to drive the ammonia over. From 0.8 to 1 cc. of the secondary alcohol was added to prevent foaming. The ammonia was blown over by a fairly strong air current into $N/10$ HCl, using 0.5 cc. of methyl red as the indicator. The aeration was continued for two and one half hours. During the time the culture was being aerated, the hydrogen ion concentration and glucose content were determined. The phosphorus content was titrated if the determination were a control determination, made that is, just after inoculation. It was not titrated in the subsequent determinations. If growth had taken place the aerobic cultures were plated on plain agar; the anaerobic cultures were counted by the Breed technique. After the ammonia aeration the acids were titrated with $N/10$ NaOH and the calculation of the ammonia nitrogen content was

made in milligrams per 100 cc. of the culture. The culture which was left in the aeration cylinder was diluted to 100 cc. in the cylinder and dilutions made from that for the amino nitrogen content. Two separate dilutions were made from which 1 cc. was placed in a test tube graduated to 25 cc. The dilutions were made in dry test tubes and all measuring of the dilution water and the culture was done with a standardized Ostwald pipette. The remainder of the procedure for the detremination of the amino nitrogen was the same as described by Folin (1922). The following day the colors were read against a standard. The two dilutions should not vary more than 2 per cent from the mean. If care is taken in preparing the dilutions the variations will not be more than 1 per cent. The readings as given later are the mean of the two dilutions.

METABOLISM OF BACTERIA

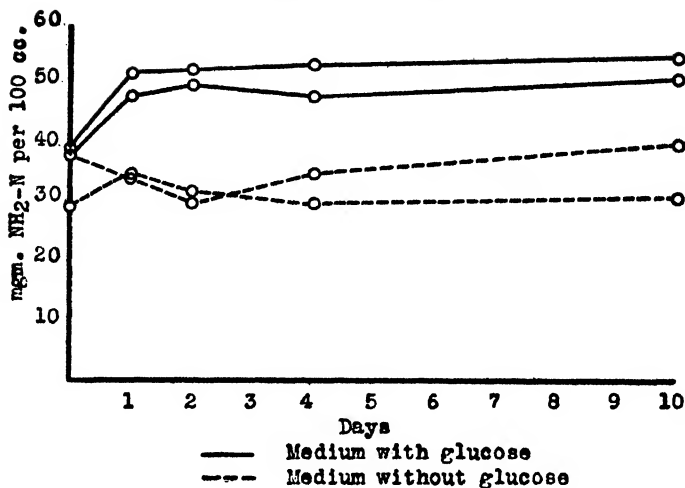
Bacterium coli

The results of the analyses of the medium during the growth of this bacterium are recorded in table 2. The experiment is divided into two series which were done at different times and are tabulated separately as series 1 and series 2. The results are shown graphically in graphs 1 to 3 inclusive.

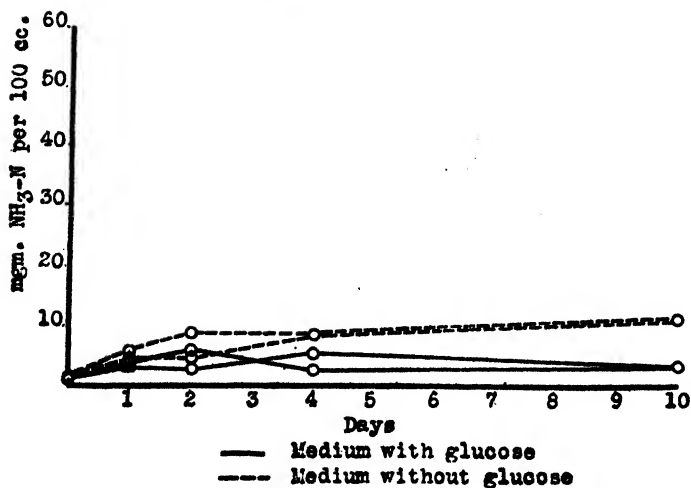
The amino nitrogen curves obtained from the medium containing glucose show higher values than from the medium without glucose. The effect of the presence of fermentable carbohydrate is apparent at once in the large increase of the amino nitrogen and the rapid change of the hydrogen ion concentration. The amino nitrogen curves of the carbohydrate free medium show fluctuating values with a final reading slightly higher than the control.

The curves of the ammonia nitrogen do not show the rapid increases that the amino nitrogen curves show. They do indicate some quantitative differences between the carbohydrate and the non-carbohydrate media, the latter being slightly higher. In general *Bact. coli* shows a strong fermentative power but does not produce large quantities of ammonia. These results agree with those of Sears (1916) and Kendall and Bly (1922) but do not

Graph No. I
Bact. coli
Amino nitrogen

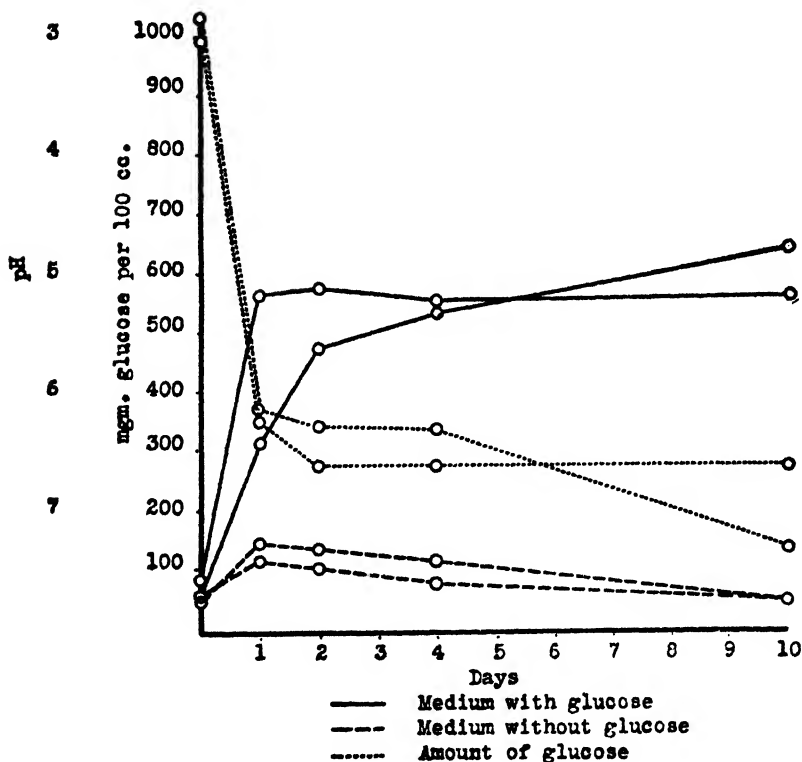


Graph No. II
Bact. coli
Ammonia nitrogen



agree with those of Waksman (1920) who found very little change, or those of Berman and Rettger (1918) who found no change in the amino nitrogen in media with and without glucose.

Graph No. III
Bact. coli
Hydrogen ion concentration
and amount of glucose



Pseudomonas pyocyanea

The results of the analyses with *Ps. pyocyanea* are shown in Table 3. The experiment is divided into two series which were done at different times and are tabulated as series 1 and 2. The graphs of the analyses are shown in graphs 4 to 6 inclusive.

TABLE 3
Ps. pyocyanea

DAY OF GROWTH									
0		1		2		4		10	
Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*									
Ammonia	11.5	1.2	8.4	13.8	14.2	16.2	16.4	19.4	40.0
nitrogen†..									
Amino									
nitrogen†..	38.8	36.7	46.0	46.0	45.0	45.0	59.4	64.2	44.7
Glucose†....	1059.0	0	1041.0	0	1090.0	0	581.0	51.0	0
pH.....	7.50	7.65	7.85	8.0	8.0	8.0	7.30	7.40	8.40
Organisms									
per cubic									
centimeter									
			530, 000, 000	138, 000, 000	670, 000, 000	270, 000, 000	780, 000, 000	260, 000, 000	150, 000, 000
Series 2‡									
Ammonia	1.1	1.1	12.4	13.6	13.6	Lost	14.1	18.6	59.8
nitrogen†..									
Amino									
nitrogen†..	31.8	31.9	47.9	40.4	47.9	40.0	55.1	64.6	51.3
Glucose†....	1020.0	0	1008.0	0	984.0	0	827.0	393.0	0
pH.....	7.65	7.70	7.75	7.75	8.00	8.15	7.70	7.65	8.20
Organisms									
per cubic									
centimeter									
			420, 000, 000	430, 000, 000	160, 000, 000	300, 000, 000	160, 000, 000	232, 000, 000	59, 000, 000

* The media in this series contained 162 mgm. P per 100 cc.

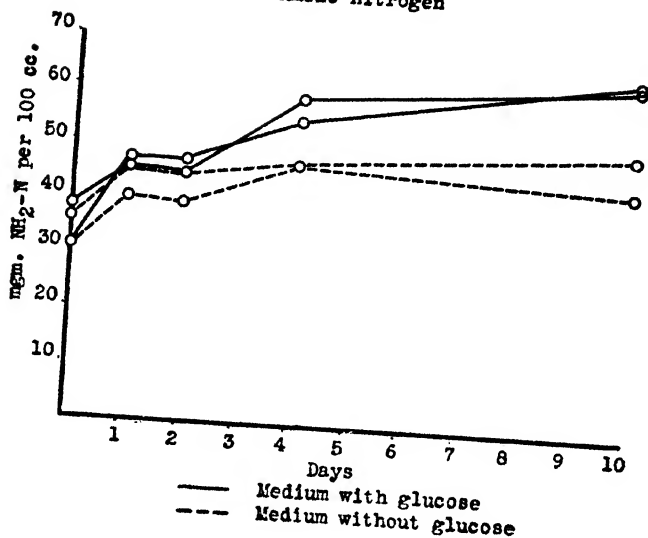
† Milligrams per 100 cc. of the medium.

‡ The media in this series contained 162 mgm. P per 100 cc.

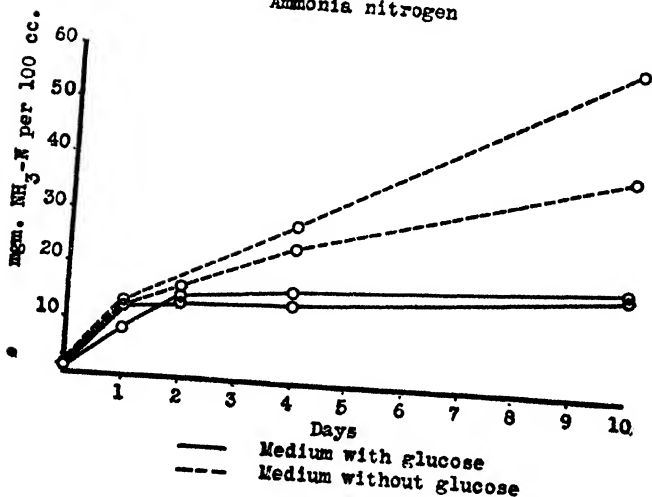
(Bottle A) This column indicates the medium with glucose.

(Bottle B) This column indicates the medium without glucose.

Graph No. IV
Ps. pyocyanea
Amino nitrogen

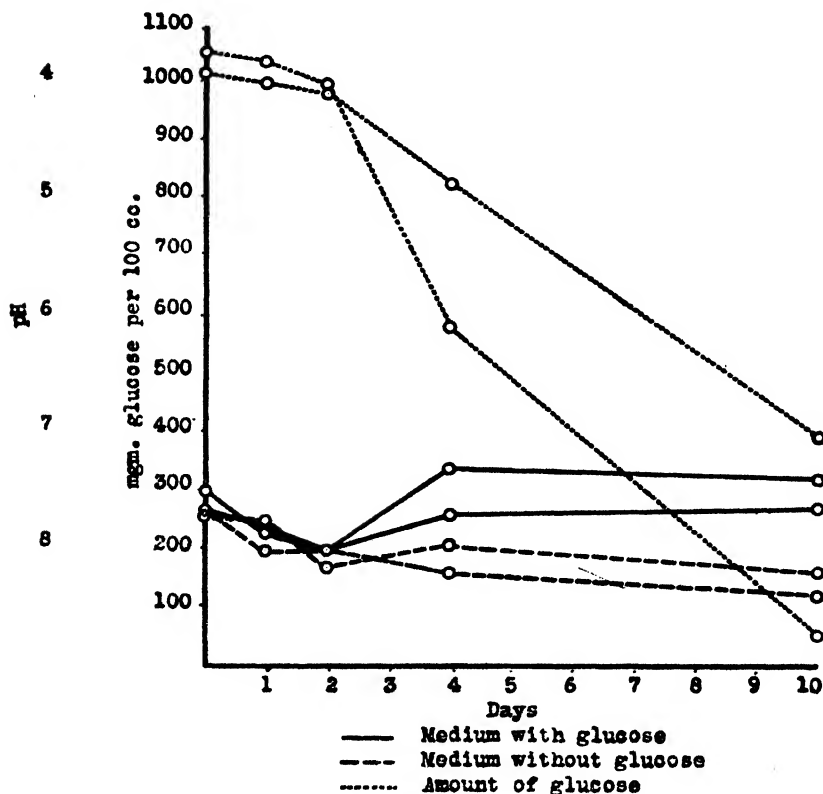


Graph No. V
Ps. pyocyanea
Ammonia nitrogen



Pseudomonas pyocyanea does not give rapid increases in the amino nitrogen in the first twenty-four hours of growth. There is a slight decrease in the amino nitrogen on the second day. At the present time there are no data available to explain this

Graph No. VI
Ps. pyocyanea
 Hydrogen ion concentration
 and amount of glucose



drop. With *Ps. pyocyanea* as with *Bact. coli* the medium containing the glucose shows a more rapid increase and a higher level in the amino nitrogen. The ammonia nitrogen shows a greater difference between the carbohydrate and the non-carbohydrate media than do the ammonia curves of *Bact. coli*.

Attention is especially drawn to the fact that the hydrogen ion concentration curves in the presence of glucose do not show a greater acidity than pH 7.0, despite the fact that the amount of glucose shows a rapid diminution. It is important to consider this fact in testing the ability of bacteria to ferment carbohydrates when acid production is used as the criterion. For example, an indicator in a growing culture of *Ps. pyocyanea* in the presence of glucose would not show acid production, and the inference might therefore be drawn that the glucose had not been destroyed, whereas, I have shown the contrary to be the case. When the curves of the hydrogen ion concentration and glucose content of *Ps. pyocyanea* and *Bact. coli* are compared the difference in the metabolism of the two bacteria is apparent at once.

Bacillus subtilis

The results of the analyses of the media with *B. subtilis* are shown in table 4. The experiment was divided into two series which were done at different times and are tabulated as series 1 and 2. The graphs of the analyses are shown in graphs 7 to 9 inclusive.

The variation in the two series in the amino nitrogen is somewhat greater than in the case of some of the other bacteria which were studied. This may be due to differences in the inoculation of the media, to the rate of growth or other factors. The presence of glucose does not appear to increase the amino nitrogen as markedly as with other bacteria tested. It is to be noted that this organism does not produce a great change in the hydrogen ion concentration in the presence of glucose. This was also noted in the case of *Ps. pyocyanea*. These two bacteria are able to destroy large amounts of glucose without producing an acid reaction. The practical importance of this observation has already been noted.

Clostridium botulinum (type A)

The results of the studies of this bacterium are shown in table 5 and in graphs 10 to 12 inclusive.

TABLE 4
Bacillus subtilis

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†.....	1.5	1.5	3.5	2.3	9.6	2.8	40.4	8.6	37.7	86.5
Amino nitrogen†.....	34.7	31.6	37.2	37.0	42.8	41.1	52.0	48.4	68.5	57.5
Glucose†.....	1020	0	986	0	833	0	753	0	367	0
pH.....	7.55	7.55	7.45	7.60	7.25	7.60	7.05	7.80	7.50	8.75
Organisms per cubic centimeter.....									Lost	Lost
			5,000,000	2,000,000	4,000,000	700,000	27,000,000	750,000,000	Lost	Lost
Series 2†										
Ammonia nitrogen†.....	1.8	1.5	2.4	3.6	4.4	15.4	14.0	43.6	84.0	100
Amino nitrogen†.....	29.7	29.5	34.7	33.8	39.7	40.9	37.5	37.5	51.6	32.7
Glucose†.....	1086	0	1000	0	880	0	568	0	28	0
pH.....	7.55	7.55	7.35	7.25	7.00	7.65	7.00	8.00	8.20	8.85
Organisms per cubic centimeter.....										
			8,600,000	1,600,000	Lost	Lost	18,000,000	3,000,000	100,000,000	34,000,000

* The media in this series contained 150 mgm. P per 100 cc.

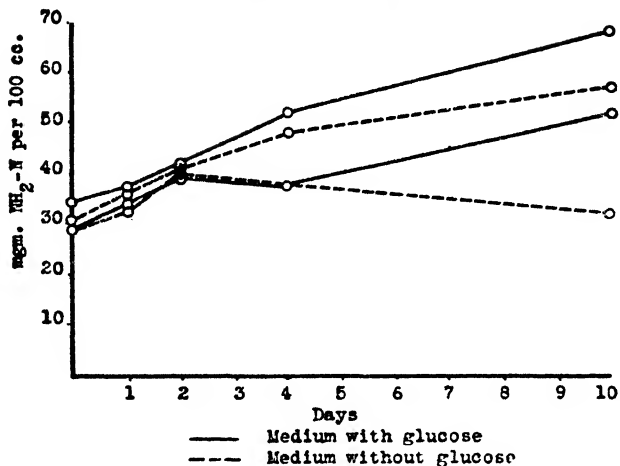
† Milligrams per 100 cc. of the medium.

‡ The media in this series contained 144 mgm. P per 100 cc.

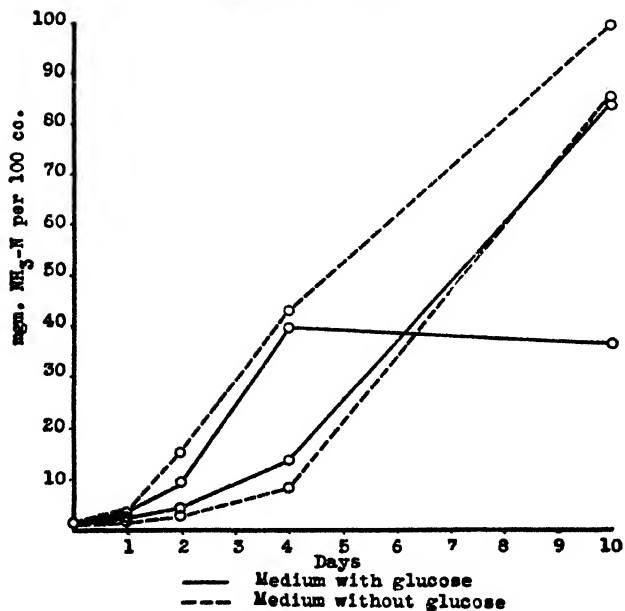
(Bottle A) This column indicates the medium with glucose.

(Bottle B) This column indicates the medium without glucose.

Graph No. VII
B. subtilis
 Amino nitrogen

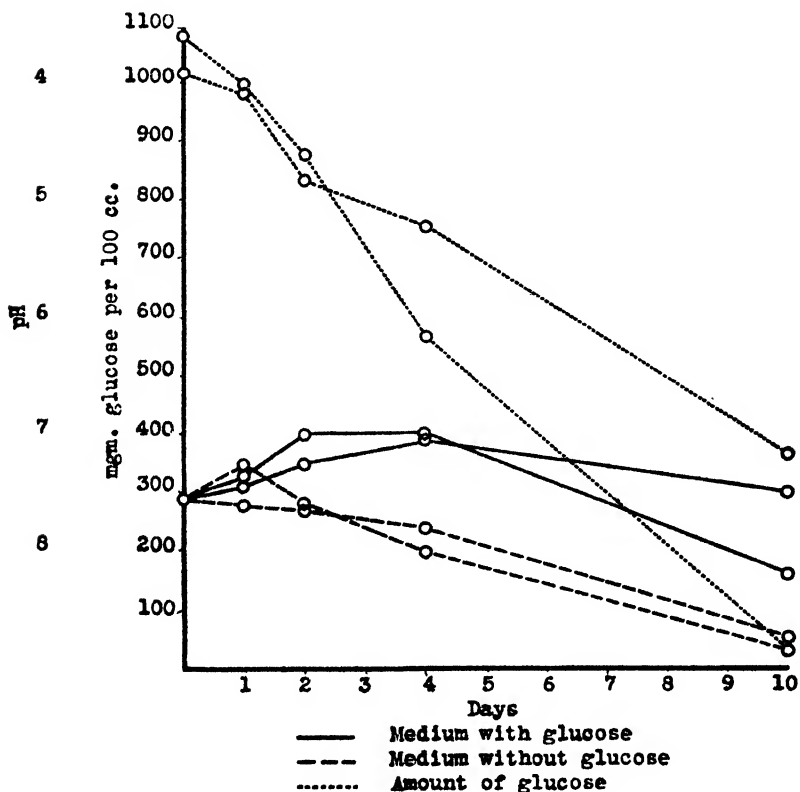


Graph No. VIII
B. subtilis
 Ammonia nitrogen



The amino nitrogen curves in the medium containing glucose do not agree very closely in total amounts of the nitrogen. However, it is to be noted that the type of curve is the same in both

Graph No. IX
B. subtilis
 Hydrogen ion concentration
 and amount of glucose



series. The medium without carbohydrate shows a decrease on the tenth day. The sharp drop in the amino nitrogen on the second and fourth days corresponds to the times when the bacteria show a definite decrease in numbers. While spores could not be demonstrated in stained smear preparations, a large number of shadow forms were noted.

The types of the ammonia nitrogen curves in the two series are very similar. The effect of the presence of the glucose is shown by these curves in that the production of ammonia is decreased. The organism is able to use glucose very rapidly as shown in graph 12.

TABLE 5
C. botulinum (type A)

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†	1.4	1.3	19.2	27.4	20.6	32.1	22.5	35.6	41.8	46.4
Amino nitrogen†	38.1	36.0	54.0	37.7	46.5	41.3	48.1	35.2	56.5	30.0
Glucose†	1054.0	0	496.0	0	367.0	0	178.0	0	33.0	0
pH	7.75	7.75	6.80	7.60	6.80	7.60	6.80	7.75	6.50	7.70
Series 2†										
Ammonia nitrogen†	2.4	1.7	18.8	25.8	18.5	29.2	20.1	49.4	22.2	58.2
Amino nitrogen†	39.2	37.5	79.7	32.8	76.5	36.5	71.7	44.2	84.9	40.6
Glucose†	1082.0	0	757.0	0	453.0	0	208.0	0	192.0	0
pH	7.50	7.50	6.30	7.65	5.65	7.70	5.55	7.70	5.40	7.70

* The media in this series contained 150 mgm. P per 100 cc.

† Milligrams per 100 cc. of the medium.

‡ The medium marked (A) in this series contained 144 mgm. P per 100 cc.; that marked (B) contained 153 mgm. P per 100 cc.

(Bottle A) This column indicates the medium with glucose.

(Bottle B) This column indicates the medium without glucose.

Clostridium botulinum (type B)

The results of the analyses are shown in table 6 and the graphs of the analyses are shown in graphs 13 to 15 inclusive.

The amino nitrogen and the ammonia nitrogen curves are very similar to those of *C. botulinum* (type A). The amino nitrogen shows a drop on the fourth day and then a gradual increase to the tenth day. The amino nitrogen curves always

show an increase in the total amount in the medium containing glucose. Kendall, Day and Walker (1922) working with six strains found an increase in three strains of *C. botulinum* and a decrease in three strains. Their results are puzzling.

TABLE 6
C. botulinum (type B)

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†.....	2.1	2.1	18.7	31.6	22.8	55.6	28.0	61.4	28.0	71.6
Amino nitrogen†.....	38.7	39.0	58.8	43.1	72.0	43.5	64.9	46.5	64.8	45.6
Glucose†.....	1091.0	0	959.0	0	806.0	0	500.0	0	250.0	0
pH.....	7.50	7.55	7.00	7.50	6.85	7.50	6.45	7.50	5.80	7.50
Series 2†										
Ammonia nitrogen.....	1.8	1.8	14.2	25.0	20.2	61.2	22.5	70.5	31.1	70.1
Amino nitrogen†.....	34.7	34.7	48.4	41.1	61.1	45.4	54.6	39.6	70.0	58.8
Glucose†.....	1041.0	0	892.0	0	463.0	0	239.0	0	42.0	0
pH.....	7.45	7.55	7.35	7.65	6.65	7.60	6.25	7.60	5.90	7.70

* The media in this series contained 144 mgm. P per 100 cc.

† Milligrams per 100 cc. of the medium.

‡ The media in this series contained 162 mgm. P per 100 cc.

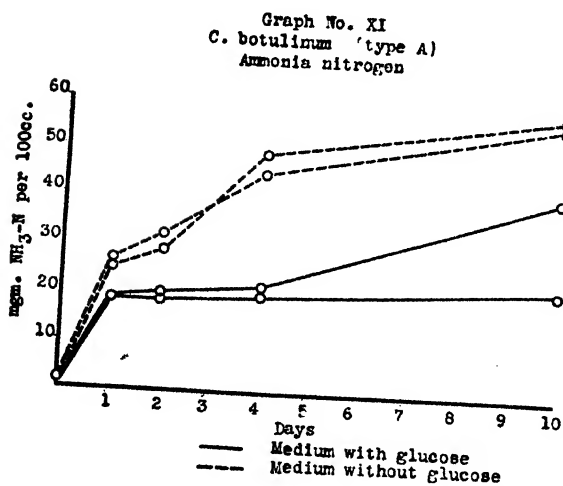
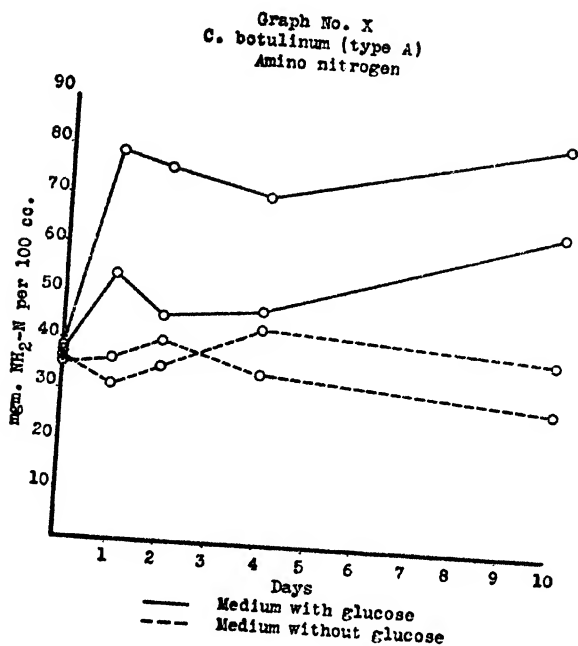
(Bottle A) This column indicates the medium with glucose.

(Bottle B) This column indicates the medium without glucose.

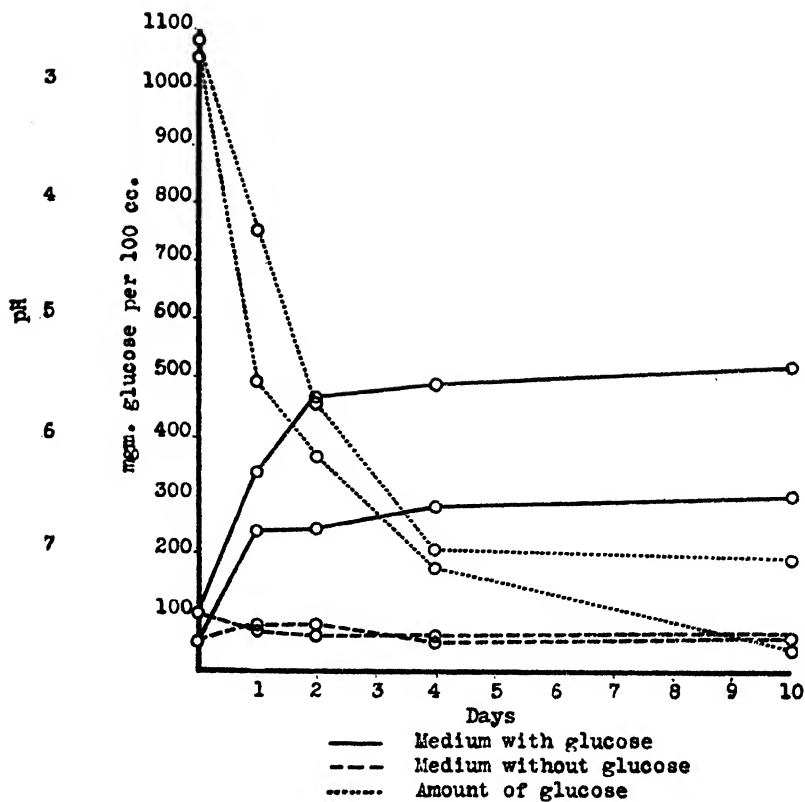
Clostridium sporogenes

The results of the analyses are shown in table 7 and in graphs 16 to 18 inclusive.

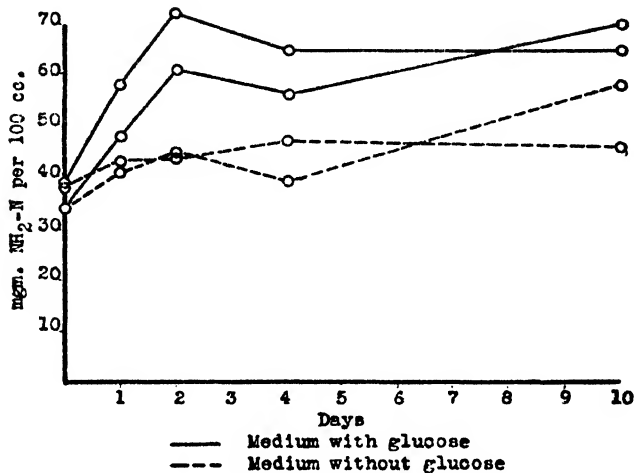
The amino nitrogen curves of this organism are very similar to those of *C. botulinum*. The presence of glucose increases the amino nitrogen and there is also a drop in the total amount on the fourth day. Wolf and Harris (1916-1917) obtained marked decreases in the amino nitrogen in a peptone solution made by the tryptic digestion of casein. In the same medium, however, an



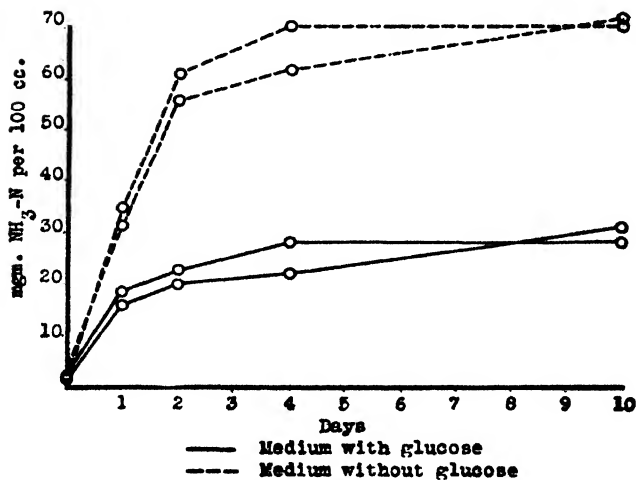
Graph No. XII
C. botulinum (type A)
 Hydrogen ion concentration
 and amount of glucose



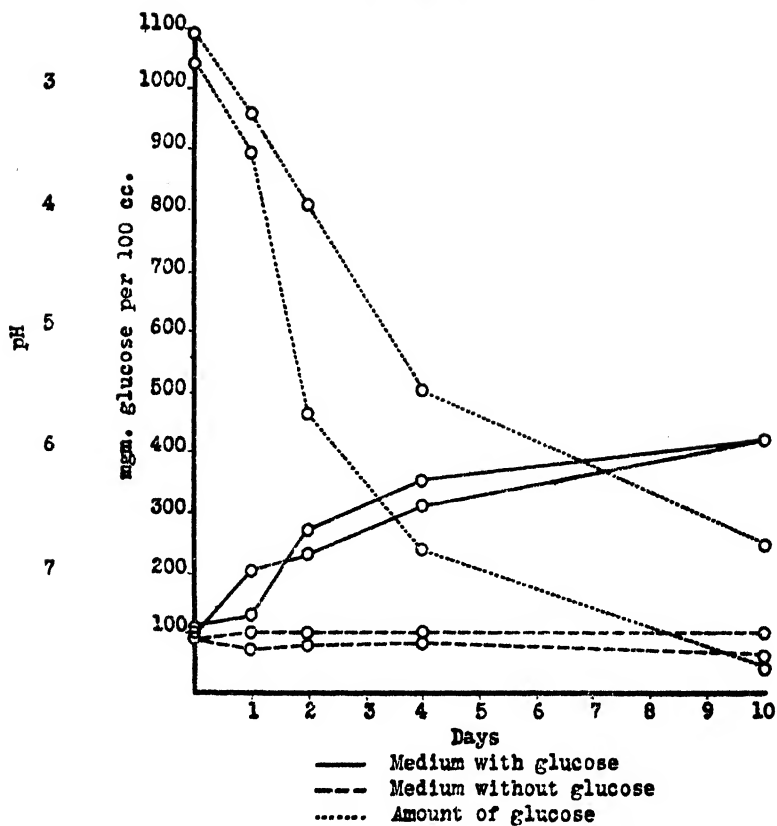
Graph No. XIII
C. botulinum (type B)
 Amino nitrogen



Graph No. XIV
C. botulinum (type B)
 Ammonia nitrogen



Graph No. XV
C. botulinum (type B)
Hydrogen ion concentration
and amount of glucose



increase was obtained when glucose was present (Harris (1919-1920)). In a minced meat medium Wolf (1918-1919 c) obtained large increases in the amino nitrogen in the absence of carbohydrate.

TABLE 7
C. sporogenes

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†.....	1.1	1.2	21.2	39.2	19.4	46.2	19.4	44.4	19.6	50.2
Amino nitrogen†.....	36.7	34.7	60.4	35.0	63.9	39.0	58.5	40.7	71.6	48.4
Glucose†.....	1096.0	0	735.0	0	505.0	0	384.0	0	312.0	0
pH.....	7.50	7.50	6.50	7.55	6.30	7.75	6.15	7.50	6.05	7.55
Series 2‡										
Ammonia nitrogen†.....	1.2	1.8	25.0	41.2	20.1	49.0	20.6	55.7	19.8	63.2
Amino nitrogen†.....	37.1	36.7	59.0	42.8	69.3	39.5	64.7	35.2	72.4	44.0
Glucose†.....	1100.0	0	771.0	0	563.0	0	416.0	0	357.0	0
pH.....	7.30	7.55	6.45	7.45	6.25	7.65	6.15	7.50	6.05	7.35

* The media in this series contained 153 mgm. P per 100 cc.

† Milligrams per 100 cc. of the medium.

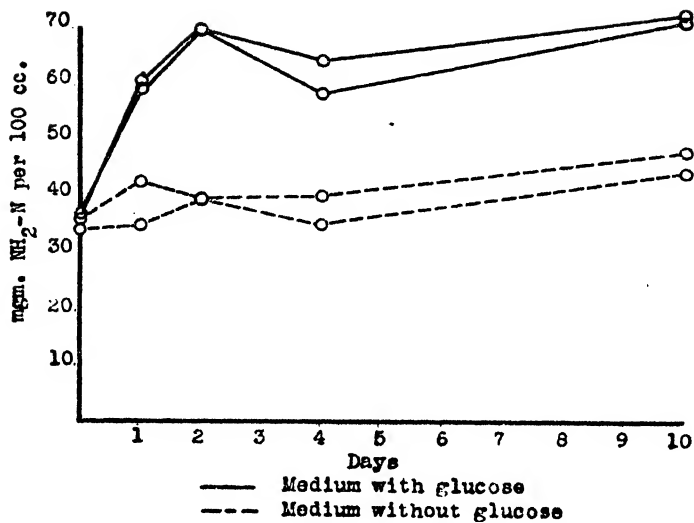
‡ The medium in this series marked (A) contained 153 mgm. P per 100 cc.; that marked (B) contained 153 mgm. P per 100 cc.

(Bottle A) This column indicates the medium with glucose.

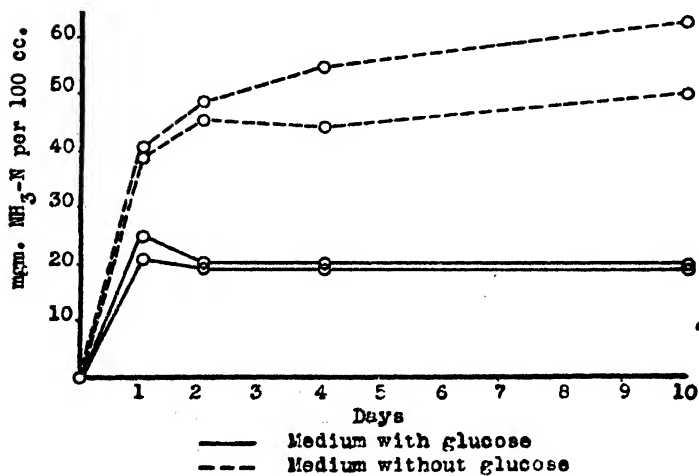
(Bottle B) This column indicates the medium without glucose.

The ammonia nitrogen in the carbohydrate medium is much lower than in the medium not containing a carbohydrate. Similar results have been recently reported by Kendall, Day and Walker (1922).

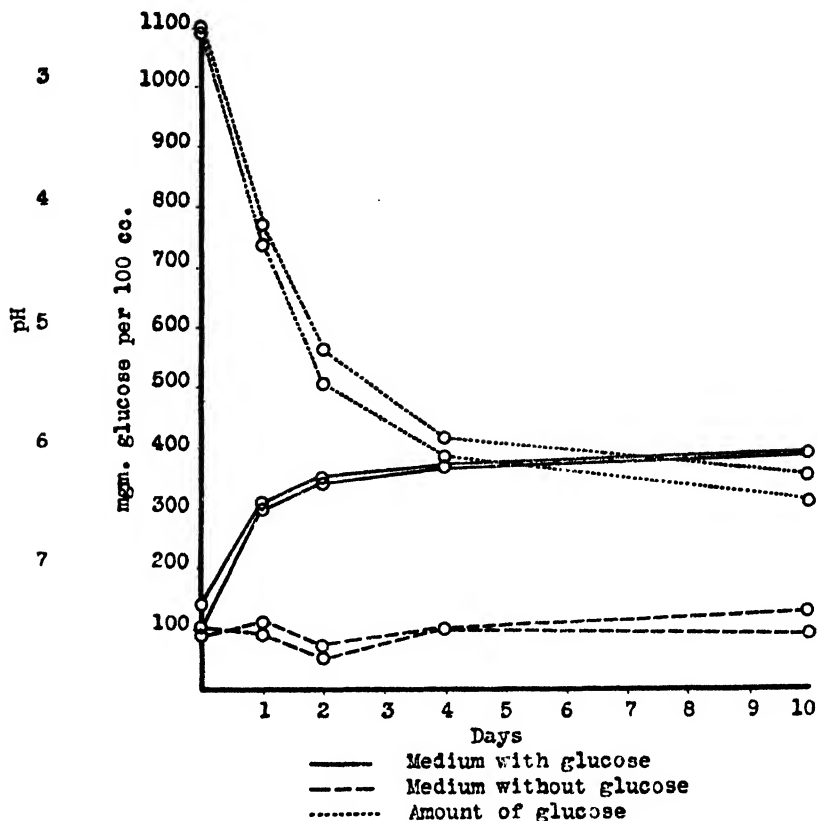
Graph No. XVI
C. sporogenes
 Amino nitrogen



Graph No. XVII
C. sporogenes
 Ammonia nitrogen



Graph No. XVIII
C. sporogenes
 Hydrogen ion concentration
 and amount of glucose



SUMMARY AND DISCUSSION

In order to study the nitrogenous metabolism of bacteria, I gave special attention to the determination of the changes in the amino nitrogen and ammonia nitrogen in growing cultures of representative bacteria. Analyses were made of the medium just after it was inoculated and again on the first, second, fourth and tenth days of growth. The usefulness of work of this type depends entirely upon the trustworthiness of the methods used.

A large part of this study, therefore, resolved itself into an investigation of the methods applicable to the conditions of the experiment.

So far as amino nitrogen is concerned, I have shown that the method of Folin (1922) gives consistent results in the presence of peptone. Thus, a sterile peptone solution gives consistent results when tested from day to day. Further, the addition of a standard glycocoll solution gives the correct increase in the amino nitrogen in sterile peptone solutions and in cultures of bacteria after growth has taken place. While it is realized that not all of the amino nitrogen may react with the reagent used in this method, it must be remembered that other methods fail to give correct readings with some of the amino acids. This is shown clearly with the simplest amino acid, glycocoll, in the Van Slyke method. It is desirable to have a method which will give the total amino nitrogen with accuracy. However, any method used in the study of bacterial metabolism should give, first of all, consistent values for the total amino nitrogen of a sterile peptone solution. Since the Folin method does give consistent values for the amino nitrogen in peptone solutions, I have adopted it as the best method available for the purpose of this investigation.

The results of my study clearly show that (1) the total amount of amino nitrogen may be increased to but a slight extent during bacterial growth in media not containing glucose, while (2) a very marked increase in amino nitrogen may take place in media containing glucose.

With the greater number of strains of bacteria used in this study there was a sharp drop in amino nitrogen on the second or fourth day of growth. The decrease takes place in the case of the anaerobic bacteria about the time they show definite autolysis and a sharp decrease in the numbers of bacteria. This decrease in amino nitrogen is not constant in the case of the aerobic bacteria. Moreover, it could not be correlated with the number of living cells.

I found the production of amino nitrogen always greater in the presence of glucose. These results do not agree with some of the results of other investigators. Berman and Rettger (1918)

found that the concentration of amino nitrogen remained unchanged in cultures of *Bact. coli* in plain as well as glucose media. They stated that the presence of carbohydrate did not affect the nitrogen metabolism. My results do not warrant such a conclusion. Kendall and his co-workers found in their extensive studies of bacterial metabolism that the amino nitrogen is changed very little during the growth of various bacteria. Kendall, Day and Walker (1922) found that three strains of *C. botulinum* produced less amino nitrogen in carbohydrate media than in non-carbohydrate media. However, the reverse was true with three other strains of *C. botulinum* which they studied. Unless a difference in the methods would account for these results, the discrepancies are difficult to reconcile. Sears (1916) working with *Ps. pyocyanea* grown aerobically showed that more amino nitrogen was produced in media containing carbohydrate than in media free of carbohydrate. On the contrary, when the organism was grown anaerobically less amino nitrogen was produced in media containing carbohydrate than in media free of carbohydrate. In view of his results it is difficult to understand Sears' conclusion that the amino nitrogen production of *Ps. pyocyanea* when grown aerobically does not differ materially from the amino nitrogen production of the same organism when grown anaerobically.

My results lead me to conclude that the amino nitrogen produced in non-carbohydrate cultures of bacteria is utilized for the growth and the energy requirements of the cells almost as rapidly as formed, and that the production of amino nitrogen in carbohydrate media is much greater than its utilization. There may be several reasons for the increased amino nitrogen production in media containing carbohydrate. Theoretically, the amino acids used for the energy requirements of the bacteria may not be necessary to the same extent if a fermentable carbohydrate be present, and therefore, the amino nitrogen would show as an increase in the medium. The presence of carbohydrate may increase the availability of nitrogenous products other than amino nitrogen which would also augment the total amino nitrogen. Again, we must consider that the increase in the hydrogen ion

concentration may influence the type of the nitrogenous metabolism. Further, there may be an increased enzyme activity. We lack sufficient data to prove whether one or all of the above factors may be concerned in the accumulation of amino nitrogen in media containing a fermentable carbohydrate.

Since the utilization of amino nitrogen may keep pace with its production under certain conditions, it is evident that the amino nitrogen cannot be used as an index of proteolysis under such conditions. However, amino acids are used by the bacteria, and therefore even if the total amino nitrogen remains unchanged, it is safe to assume that there must have been a certain amount of proteolysis to replace the amino nitrogen utilized. The direct proof can not be obtained from the determinations of total amino nitrogen. If the amino nitrogen were not used in the metabolism of bacteria, an increase would always be expected which could be taken as a quantitative index of proteolysis. From the above considerations it is concluded that amino nitrogen may be taken only as an approximate index of proteolysis in bacterial cultures which show a material increase in total amino nitrogen. This is shown clearly in media containing a carbohydrate. In media not containing a carbohydrate the amount of amino nitrogen cannot be taken as an index of proteolysis except in the cases where the amino nitrogen is increased. In any case, it is clear that the amino nitrogen in bacterial cultures can not be taken as a quantitative index of proteolysis.

The total amount of ammonia produced depends upon many factors, especially the composition of the medium (presence or absence of carbohydrate) and upon the species of organism used. *Bact. coli* produces very little ammonia when compared to *B. subtilis*, *Ps. pyocyanea* and the anaerobes, *C. botulinum* and *C. sporogenes*. In all of my experiments in this study the presence of a fermentable carbohydrate has resulted in a lessened production of ammonia when compared to a non-carbohydrate medium. The cause of this decreased rate of production of ammonia has not been definitely proven. It has been claimed by Kendall and Walker (1915) that this decreased production of ammonia is due to a decreased rate of proteolysis. Gordon (1917) has

shown that *Bact. coli* is able to use certain ammonium salts, for example, ammonium chloride, as the only nitrogen source when a carbohydrate is present. Therefore, the fact that ammonia is decreased in carbohydrate media does not necessarily indicate a smaller amount of proteolysis; it may indicate a greater utilization of the ammonia. Further, the increase in the hydrogen ion concentration may influence the type of metabolism and thus affect ammonia production.

If ammonia production is taken as an index of proteolysis, as proposed by Kendall and Walker (1915), their conclusion that there is less proteolysis in media containing a carbohydrate would be justified. However, if the production of amino nitrogen is taken as an index of proteolysis, the fact that there is a large increase in the amino nitrogen in carbohydrate media would indicate that there is greater proteolysis. Since the number of bacteria in a medium containing carbohydrate is many times greater than in a medium not containing carbohydrate, it is evident that the total nitrogenous metabolism in the presence of sugar must of necessity be greater than in the absence of carbohydrate. Ammonia if it be taken as an index would indicate a decreased total nitrogenous metabolism, therefore, under these circumstances, it seems justifiable to conclude that ammonia production in bacterial cultures is not a reliable index of proteolysis. The change in the rate of ammonia production in media containing carbohydrate may indicate the extent of either deamination or utilization of ammonia or the balance of the two processes. While amino nitrogen may not be an absolute index of proteolysis it does indicate, contrary to ammonia, an increase in the rate of nitrogenous metabolism in bacterial cultures in the presence of carbohydrate.

Since the cultures show a large increase in the total amount of amino nitrogen in media containing glucose, the suggestions that the proteolytic enzyme was inactivated by glucose as proposed by Fischer (1915) or that glucose prevented the formation of the proteolytic enzymes as proposed by Kendall and Walker (1915) and Jones (1916) do not seem plausible.

The organisms studied divide themselves into two groups with reference to hydrogen ion concentration. One group, including *Bact. coli*, *C. sporogenes* and *C. botulinum*, shows a large reduction of glucose with a markedly increased production of acid as determined by the hydrogen ion concentration. The other group, which includes *B. subtilis* and *Ps. pyocyanea*, destroys approximately the same amount of glucose without the production of acid, as determined by the hydrogen ion concentration. It is important to note that the medium in which both these groups of bacteria were grown had approximately the same composition with reference to the initial amount of glucose and buffer content.

It is therefore apparent that some organisms have the power to destroy glucose without markedly increasing the hydrogen ion concentration; thus indicating a different type of carbohydrate metabolism. If the hydrogen ion concentration is not increased the inference commonly drawn is that the glucose is not destroyed. My data show that the contrary may take place and the inference is therefore not justified. The conclusion is clear, then, that the hydrogen ion concentration of cultures of different bacteria in media containing approximately the same amount of buffer does not necessarily prove whether the carbohydrate has or has not been destroyed.

CONCLUSIONS

The presence of glucose in peptone media increases the rate of production of amino nitrogen in growing cultures of *Bact. coli*, *Ps. pyocyanea*, *B. subtilis*, *C. botulinum* and *C. sporogenes*.

The amino nitrogen found in bacterial cultures may be taken as an approximate index of proteolysis under certain conditions.

The Folin method for the determination of amino nitrogen is applicable in the studies of bacterial metabolism in peptone media.

The ammonia found in bacterial cultures is not a reliable index of bacterial proteolysis.

The presence of a fermentable carbohydrate in bacterial cultures affects the nitrogenous metabolism as judged by the total amino nitrogen and the total ammonia nitrogen.

The rate of production of amino nitrogen or ammonia nitrogen indicates different types of metabolism of bacteria.

Some bacteria destroy glucose without a marked increase in the hydrogen ion concentration. Therefore the hydrogen ion concentration may not be an index of the destruction of glucose in bacterial cultures.

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DEVICE FOR TUBING COOKED MEAT MEDIUM

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The use of meat media for anaerobic as well as aerobic growth has become more or less general since the war. I discussed some of its advantages in routine laboratory work in 1919.¹ Tubing the medium has proved rather time consuming and the upper portion of the tubes gets smeared with particles, which are troublesome to remove and which give an untidy appearance to the finished product. To overcome these difficulties, I have made use of the ordinary automobile grease gun with complete success and the tubing of meat medium has become clean and rapid. The front cap and nozzle are unscrewed and the barrel filled with meat in the same way as it is filled with grease. There are several forms of grease guns with side openings which are probably more convenient for filling than the one I have used. The nozzle must not have too small a diameter and must reach past the middle of the tube. The fluid portion is added to the meat particles through the ordinary tubing funnel. In large establishments an apparatus like the sausage filling machine may be used.

In routine work I have found it very convenient to buy the ground meat from the butcher, make it up with an equal quantity of water, sterilize, neutralize, allow to stand over night, skim off the fat, tube as above, adding the fluid last, and autoclave at 120°C. for thirty minutes. I prefer skeletal to heart muscle since the latter forms particles which are too hard when sterilized and I have found brain makes a medium which is too soft.

¹ Jour. Bacteriol., 1919, 4, 149.

This meat medium keeps anaerobic for days and weeks and there is no necessity to boil and cool just before use as was formerly believed (unless for very special purposes), nor need the surface be covered with oil or paraffin. Complete mixing of the seeded material with the meat particles is the most important point.

Its simplicity of preparation, tubing and sterilizing, its wide range of use in growing bacteria, and its keeping qualities should make cooked meat the medium of choice for routine bacteriologic studies.

BACTERIOPHAGE PHENOMENA¹

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INTRODUCTION

During the past four years an increasing number of articles have appeared in the bacteriological literature on the so-called "bacteriophage phenomena." It seems advisable, therefore, to give a brief historical review of the data which have accumulated to date and the various theories advanced in regard to this subject, before reporting our own experiments. The fundamental discoveries will be stated first and then the more recent detailed experiments will be discussed as they bear upon the particular point under consideration.

General interest was first attracted to this subject by the work of d'Herelle which was published towards the latter part of 1917. It is now, however, conceded by the majority of workers in this field, that the first observations of this phenomenon were made by Twort in 1915.

Twort (1915) was working on the problem of cultivating filtrable viruses. In some of his experiments he used glycerinated calf vaccinia. He inoculated agar tubes with the vaccinia before the glycerine had sterilized it completely, and found that the colonies of yellow and white micrococci that grew out, showed what he described as "watery" areas. These areas could not be sub-cultured. He found, furthermore, that if the water of condensation from these tubes was plated, colonies of the micrococci developed which also became transparent. If he touched a normal colony of the micrococci with some of this "transparent material," the normal colony would become "watery." The

¹ Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University, June, 1922.

"transparent material" was still active in high dilution (1:1,000,000), and remained so after passage through a Pasteur Chamberland filter. All attempts to sub-culture the filtrate proved negative. If the filtrate of the transparent material was added to an agar slant which was then inoculated with a normal culture of the micrococcus, the organism would start to grow, but the growth soon become translucent. The filtrate used in higher dilution, produced only a small number of transparent spots which appeared at various points of the culture. Twort found that the transparent material could be transmitted from one young culture to another indefinitely. He also observed that these clear areas appearing on slant would in time be overgrown, but that the transparent material was still active when added to a new, growing culture of the micrococci. Thus, Twort, in 1915, stated all the fundamental characteristics of this phenomenon which have been confirmed in practically every detail in connection with all the different "bacteriophages," active against a wide variety of organisms, that have been discovered since.

To summarize briefly the characteristics of the "transparent material" isolated by Twort from micrococci growing out from calf vaccinia:

1. It can be separated from the organisms from which it is derived by filtration.
2. It can be transmitted indefinitely from one culture to another.
3. It is most active against young actively growing organisms.
4. It has no action on dead organisms.
5. It cannot be transmitted without the presence of organisms in any media.
6. It is active in high dilution, 1:1,000,000.
7. It is fairly heat resistant, 60° for one hour being necessary to destroy it.
8. The action is not hastened by anaerobiosis.
9. It increases in quantity when allowed to act on a culture.
10. In old cultures the activity of the transparent material is diminished or impeded.
11. It acts to a less degree on closely related organisms, such as Gram positive cocci isolated from boils.
12. It does not act on organisms unrelated to the micrococci—such as *Bact. coli*, streptococci, tubercle bacilli, yeasts.

Twort in concluding his article considers most of the hypotheses as to the nature of this substance, that are still under discussion today. Twort assumes that if derived from the organism itself, it may be either

a stage in the life history of the organism which is filtrable and will not grow on any media and stimulates other cultures to pass to the same stage; or it may possibly be an enzyme which is secreted by the bacteria and leads to their own destruction, a process during which the amount of the enzyme is increased. Twort also discusses the possibility that this material might be a filtrable virus which is present as a contamination. Animal inoculation was absolutely negative, this definitely ruling out the vaccinia, and Twort found no very good reason for considering it a non-pathogenic virus. He concluded that it might best be regarded as "an acute infectious disease of micrococci."

Twort also mentions a bacillus not related to the *Bact. coli* group, isolated from cases of infantile diarrhea which had the property of "dissolving itself."

Twort's work did not attract very much attention, and it was not until d'Herelle published a series of papers on what is now generally considered the same phenomenon, that Twort's contributions to the subject were seriously studied.

D'Herelle (1917) was studying the effect of the addition of stool filtrates obtained from dysentery cases on the growth of the Shiga dysentery bacillus. In his original experiment, he obtained daily specimens of feces from a dysentery patient and filtered them, adding the filtrate to fresh Shiga cultures. He reported this experiment until one day when the patient had reached the convalescent stage, he observed that the turbid Shiga culture had after an interval become clear, subsequent to the addition of the last stool filtrate. He next found that the addition of a small quantity of this cleared culture to another turbid young Shiga culture would dissolve the second culture, and that he could repeat this process indefinitely from one dissolved culture to another. In one instance, he transmitted the lytic principle originally obtained from a stool filtrate, through 935 culture generations, in each case adding only a minute quantity of the dissolved culture to the next tube, so that the original stool filtrate which started the lysis was soon eliminated in the successive dilutions. The dissolved cultures were not always completely sterilized. If sub-cultured, a small number of colonies would be obtained, the character of which will be described in detail below.

The lytic material isolated by d'Herelle has all the characteristics of Twort's "transparent material" outlined above. The

fact that the stool filtrates and their derivatives were active only against living bacteria and could be carried on indefinitely from one culture to another convinced d'Herelle that he was dealing with an ultramicroscopic filtrable virus which was parasitic on bacteria, to which he gave the name "*Bacteriophagum intestinale*." This name "bacteriophage" has now come into general use for phenomena of this type, irrespective of the source of the material which first starts the lytic process or the particular organism against which it acts, and in spite of the fact that the theory which the name implies, is not accepted by the majority of investigators at the present time.

D'Herelle does not believe that phenomena described by him relating to the typhoid-dysentery-coli group are the same as those described by Twort, and since repeated by Gratia and Callow with staphylococci and vaccine virus. D'Herelle states that the two phenomena cannot be the same because of the differences in heat resistance. Twort in his original article says that his "transparent material" resists a temperature of 52° for one hour, but is destroyed at 60° for one hour. D'Herelle considers that one of the essential criteria of true bacteriophage action is the fact that the bacteria which persist after the dissolving action of the bacteriophage has taken place, are killed at a temperature which does not destroy the bacteriophage. For instance, the Shiga bacillus is killed at a temperature of 60°C. for thirty minutes, whereas the particular bacteriophage which d'Herelle tested was only destroyed at 65°C. But, as Gratia (1921) has pointed out, the heat resistance of the bacteriophage is not a fixed property and depends upon the particular culture against which the bacteriophage is tested. Thus, in Gratia's experiment, a bacteriophage heated at 56°C. for thirty minutes lost its activity against staphylococcus A, but retained it for staphylococcus B, and regained it for strain A. Callow working in our laboratory has isolated a bacteriophage from vaccine virus active against staphylococcus which is diminished but not completely destroyed at 75° for thirty minutes. Thus, the heat resistance of bacteriophages is a variable property which depends not only on methods of testing, but also on the

way the bacteriophage is obtained. Any one who has compared the action of a staphylococcus bacteriophage isolated from vaccine virus and a dysentery or typhoid bacteriophage cannot doubt that the same phenomenon is involved in both cases. Certain minor differences do exist, but they can probably be attributed to the biological differences between staphylococci and members of the typhoid-colon-dysentery groups, since these organisms serve as the indicators upon which we depend for evidence of bacteriophage action.

D'Herelle has recently published a book on "Le Bacteriophage" which contains the results of four years of experimental work on this subject. Before going on to the theoretical aspects of the subject, it may be well to enumerate a few of the most important observations made by him, in addition to the fundamental principles which apply to all bacteriophage phenomena outlined above, from Twort's work.

(1) Filtrates from the stools of typhoid and dysentery convalescents in a large percentage of cases contain a lytic principle which has the power of dissolving or inhibiting the growth of typhoid or dysentery bacilli, and which can be transmitted in series indefinitely.

(2) The lytic principle isolated from stool filtrates is usually not absolutely specific, but is active in most cases against two or more members of the dysentery, typhoid or colon group.

(3) The activity of the lytic agent is often feeble on first isolation from the stool filtrates, but rapidly becomes more active when transmitted in series. Not only in subsequent generations, is the lysis more rapid and the amount necessary to produce lysis from one tube to the next much smaller, but also the range of activity may be extended, so that a filtrate which on first isolation is active only against Shiga and Y dysentery bacilli may after three or four passages with Shiga bacilli show activity against typhoid bacilli as well. It is also possible in some instances to make the lytic principle active against organisms which are not attacked at first, by successive contacts with these bacteria.

(4) Lysis occurs most readily with young cultures of bacteria which are multiplying rapidly. Antiseptics or temperatures which in anyway retard the growth of the bacteria, although they do not in any way lessen the activity of the lytic principle itself, interfere with the lytic action.

(5) It is impossible to transmit the bacteriophage in series in filtrates of broth cultures of susceptible organisms. The products of bacterial growth are not sufficient, the living multiplying bacteria are absolutely necessary.

These, briefly, were the most important observations made by d'Herelle at the time that we began working on this subject in November, 1920. His book which reached this country during November, 1921, contains many new facts which will be discussed below. D'Herelle in his first article published in September, 1917, claimed that the lytic principle isolated by him from dysentery stools and called by him bacteriophage, was an ultramicroscopic living organism parasitic on bacteria, and has maintained this point of view. He considers that the bacteriophage is a normal inhabitant of the intestinal tract, where it is parasitic on *Bact. coli*. During the course of intestinal disease, the bacteriophage becomes parasitic on the invading organism. D'Herelle believes that there exists only one bacteriophage which by processes of adaptation is capable of attacking different organisms with which it comes in contact.

The first investigator to question the living nature of the bacteriophage was Kabeshima (1920). He advanced the following reasons for thinking it a ferment.

(1) An extremely small amount of lytic filtrate is sufficient to dissolve a fairly large number of bacteria.

(2) The lytic filtrate is still active after standing for four years in a sealed tube.

(3) It resists heating (moist) at 70°C. for one hour.

(4) It resists the action of chloroform, toluene, alcohol, ether, carbolic acid, and acetone.

Kabeshima originated the following method of isolating bacteriophage: to 1 volume of lytic filtrate add 3 volumes of acetone. Let the mixture stand at room temperature for forty-eight hours, shaking at intervals. Evaporate the acetone. A yellowish powder remains. The lytic action of this powder according to Kabeshima, is stronger than that of the original lytic ferment.

Kabeshima explained the increase of bacteriophage which occurred when the lytic principle was brought in contact with a susceptible

organism in the following way: In the course of disease, a certain gland in the digestive tract in an effort to digest the invading bacteria, produces a catalyst. This catalyst activates a proferment contained in the bacteria, liberating a ferment which causes the organisms to autolyze. In the next generation this ferment acts as the catalyst and activates the proferment in the new bacteria, again causing them to autolyze, etc. Kabeshima does not state whether he considers the original catalyst and the liberated ferment identical, but their mode of origin is so different that this does not appear likely. In any case, the catalyst and the ferment have both the power of activating the proferment present in bacteria.

The next most important contribution to the subject was made by Bordet and Ciuca in a paper published October, 1920. Bordet was the first worker to demonstrate that a lytic principle such as that described by d'Herelle could be obtained without starting from a stool filtrate.

By injecting a certain strain of *Bact. coli* into a guinea-pig intraperitoneally, three or four times at intervals of a few days, Bordet and Ciuca obtained an exudate containing a large number of leucocytes and a few living bacilli. He added several volumes of broth to this filtrate, allowed it to stand at room temperature a few hours or a day and then filtered. This filtrate when added to a normal culture of the colon bacillus produced lysis transmittable in series.

D'Herelle in his early experiments described bacteriophages of such great activity that they sterilized the cultures completely, and no growth was obtained when the dissolved cultures were plated. Bordet and Ciuca were the first investigators to show that in most cases bacteriophage action did not completely sterilize the culture that had been dissolved, but that if such a culture were plated a small number of colonies would develop that presented peculiar characteristics. They develop more slowly than normal colonies and have a tendency to be irregular in outline, and grow discreetly. If these colonies are transplanted to broth, the broth never becomes definitely turbid, and the supernatant fluid of this broth culture, if added to a normal colon culture, has lytic power. In other works, certain colonies that grow out after lysis has taken place, carry the lytic power. Border and Ciuca found that if these cultures were sub-cultured on agar for several generations, they would grow luxuriantly, and often present a glassy mucoid type of growth. They found that these mucoid coli were more virulent

and less easily phagocyted than normal *Bact. coli*. They also reported that if a guinea pig received an M.L.D. of *Bact. coli* and then received an injection of dissolved culture heated at 58° for thirty minutes, this animal survived, whereas the control died in eight hours.

Bordet and Ciuca like Kabeshima, did not agree with d'Herelle that bacteriophage action was due to a living virus. They interpreted this phenomenon as a hereditary tendency for a given culture to autolyze, acquired during exposure to unfavorable circumstances, such as are produced by the leucocytic response in the peritoneal cavity of the guinea pig.

Bordet and Ciuca (1921a) were the first workers to produce an antilytic serum by inoculating rabbits with increasing amounts of dissolved culture filtrate. The serum of a rabbit treated in this way, was able, according to Bordet and Ciuca, to neutralize the lytic action of their bacteriophage completely. If equal quantities of the lytic agent and the lytic immune serum were added to a culture of *Bact. coli*, normal growth would be obtained, whereas, if in a parallel tube the lytic agent alone was added, no growth occurred. Bordet and Ciuca also showed that the antilytic serum could change a lytic irregular colony such as has been described above, back to a normal colony in the following way. Two agar slants were inoculated, respectively, with 7 drops of the antilytic serum and 7 drops of normal rabbit serum, and incubated overnight. The following day these two slants and one agar slant to which nothing had been added, were inoculated with a lytic colon culture. The slant which had been in contact with the antilytic serum showed confluent, normal growth after eighteen hours; the other two slants, sparse growth of irregular colonies. If these three slants were transplanted to broth, the *Bact. coli* that had grown in the presence of the antilytic serum would cloud the broth uniformly, whereas the broth transplants from the other two slants would remain clear. Thus, the antilytic serum could inhibit or prevent the inheritance of the lytic quality.

The important contributions made by Bordet and Ciuca are:

- (1) That a lytic agent transmittable in series could be demonstrated in the leucocytic exudate obtained by repeated injections of *Bact. coli* into the peritoneal cavity of a guinea pig.

- (2) That after lytic action had taken place on a given culture, certain colonies developed on subculture which carried the lytic agent in subsequent generations.

- (3) That the lytic agent was antigenic; the antilytic serum was able to prevent the action of the lytic agent, and also convert a lytic-bearing colony to a normal colony.

Thus, three fundamentally differing theories had been advanced when we began working on the subject of bacteriophage. D'Herelle attributed these phenomena to an ultramicroscopic virus parasitic on bacteria, a normal inhabitant of the intestinal tract of man and animals; Kabeshima, to a new type of enzyme which attacked living cells and was liberated in increased quantity from the substrate on which it acted, and lastly, Bordet and Ciuca, to an acquired hereditary tendency to autolyze, induced by the action of leucocytes on bacteria.

I. OBSERVATIONS ON THE ISOLATION OF THE LYTIC AGENT BY THE METHODS OF BORDET AND CIUCA, AND OF D'HERELLE

Bordet and Ciuca's experiment in which they produced *Bact. coli* bacteriophage experimentally rather than isolating it from stool filtrate in the course of a pathological condition, seemed of the utmost importance from the point of view of explaining the nature of the lytic agent. Our first attempt was, therefore, to duplicate the result of these workers.

Ten guinea-pigs were injected with 10 different strains of *Bact. coli* and the exudate treated exactly according to the directions of Bordet and Ciuca. In no instance, however, was any lytic activity of the exudate demonstrable. Wollstein (1921) repeated Bordet and Ciuca's original experiment, and recommended injecting the guinea-pig once instead of three or four times. We have been able in 2 guinea-pigs to produce a lytic exudate by a single intraperitoneal injection of typhoid bacilli.

It is the opinion of all workers, however, that this method of obtaining the bacteriophage principle is extremely unreliable, and that the factors that determine its development are not understood. Bail has recently reported that he has been able to obtain lytic exudate by the injection of Shiga bacilli, using a single dose of such a size that the pig would require two or three days to die. Gratia has also used this method successfully with staphylococci, but states that he cannot control the various factors in the experiment and that he cannot be sure of obtaining a lytic exudate every time.

D'Herelle has never been able to repeat this experiment of Bordet and Ciuca. He considers the development of lytic exudates as due to the permeation of the intestinal wall by a bacteriophage which preëxisted in the intestine of the pig, as a result of the irritation due to infection in the peritoneal cavity. He attributes the irregularity of these experiments to the fact that the presence of a bacteriophage in the intestine of a particular guinea-pig active against, or at least capable of adaptation against the strain injected, is purely accidental. D'Herelle does not include any guinea-pigs in his series investigating the presence of bacteriophage in the feces of normal animals. Recently, however, Lisbon and Carrère (1922) have attempted to show that the origin of the bacteriophage is in leucocytes rather than in the intestine, but their experiment is not clear cut. They produced sterile abscesses in a series of 5 animals by the injection of sterile petroleum. They were able to isolate a lytic agent active against Shiga dysentery bacilli from the pus thus obtained, in every case. They state, however, that the feces of 4 out of 5 animals tried contained a bacteriophage active against Shiga bacilli. The feces of these animals were not examined before the abscesses were induced, so that it is difficult, since we are dealing with an extremely diffusible substance, to say whether or not the bacteriophage originated in the leucocytes or in the intestine. Since this method of obtaining a bacteriophage principle was first described by Bordet and Ciuca, so many simpler methods of starting the lytic process have been discovered that give greater promise of solving the nature of these phenomena, that it has not seemed worth while to analyze the conditions obtained by peritoneal injections more closely. The fact that leucocytes may play some rôle in starting the lytic process is also suggested by the fact that staphylococcus bacteriophage is so easily isolated from green calf vaccine virus and also, as shown by Callow, from boils.

We may state, however, that while we were unable to obtain a lytic exudate by the method of Bordet and Ciuca with 11 strains of *Bact. coli*, we succeeded in two instances when typhoid bacilli were injected.

Isolation of a typhoid bacteriophage by the d'Herelle technique

A bacteriophage principle was isolated by the technique discovered by d'Herelle, from the stool of a typhoid convalescent, Ida Olsen, sent to me by the courtesy of Dr. Krumwiede of the Research Laboratory of the Health Department.

The stool was plated and typhoid bacilli isolated. Nothing abnormal was noted in the appearance of the typhoid colonies. They agglutinated readily on the slide in a typhoid immune serum in a dilution of 1:100. A small particle of feces was emulsified in broth and incubated overnight. On the following day twice the volume of broth was added, the emulsion was centrifuged, and filtered through a Berkefeld filter. This filtrate was tested out in the following way, against the homologous typhoid strain, no. 18.

(a) 2 cc. extract broth + 1 loop of four hour broth culture no. 18 + 0.5 cc. stool filtrate.

(b) 2 cc. extract broth + 1 loop of four hour broth culture no. 18 + 0.5 cc. sterile salt.

These tubes were incubated for three hours and then left standing overnight at room temperature. At the end of this time the control tube (b) was definitely cloudy, and the tube with the filtrate (a) still clear. One half of the tube (a) was now added to 2 cc. extract broth + 1 loop of a young broth culture of typhoid no. 18. This second tube also failed to show growth as compared with the control on the following day. The inhibitory property of tube (a) could be carried on from one generation to another indefinitely. It was also found that one of these inhibited tubes could dissolve a young typhoid culture which was definitely turbid, clarifying it completely. The lytic and inhibitory action was stronger after a few generations in contact with typhoid bacilli than it was in the original stool filtrate.

This lytic principle which has been isolated for over a year and a half at the present writing and with which most of the work described in this paper was done, differs in no essential from the many bacteriophages described by d'Herelle. The Olsen bacteriophage acts non-specifically within the typhoid, colon, dysentery group. It is lytic and inhibitory for Shiga, Mt. Desert and Flexner dysentery bacilli. In the original experiments when the Olsen bacteriophage was not far removed from

the original stool filtrate, it showed no action on paratyphoid A or B. But when tried again recently against these cultures, it was active against both of these organisms. The Olsen bacteriophage has no lytic action against laboratory stock strains of *Bact. coli-communis* and *communior*. It does, however, act against a recently isolated *Bact. coli* which occurred in the stool culture of a typhoid carrier referred to below. Typhoid strains show the greatest variation in susceptibility to lytic agents. The Olsen bacteriophage has acted on most of the typhoid cultures tried, whether a stock culture (Mt. Sinai) or recently isolated strains, with the exception of the Rawlings strain. It has recently been possible to obtain a susceptible variant of the Rawlings culture (see below). D'Herelle states that from the point of view of susceptibility to lytic agent, both the typhoid group and the colon group must be regarded as heterologous, whereas the members of dysentery groups are all fairly susceptible. The Olsen bacteriophage has no action against a stock strain of cholera or against any Gram positive organism that has been tried, such as the pneumococcus or staphylococcus. No systematic attempts to acclimatize the Olsen bacteriophage to organisms against which it was not active have been made. The fact that the range of activity of a particular bacteriophage could be extended was first shown by d'Herelle and has since been repeated by other workers, notably Bordet and Ciuca (1921b). This adaptation of the bacteriophage is of theoretical importance and must be taken into account in any theory that is advanced.

Types of colonies developing after lysis

The Olsen bacteriophage never completely sterilized the cultures which it dissolved or inhibited. On subculture it was found that a small number of colonies developed which were in most cases of two types; one a typical round typhoid colony, the other an irregular jagged colony. The latter type if fished into broth, produced little or no clouding and the supernatant fluid could be shown to contain the lytic agent in the same way that Bordet and Ciuca had demonstrated in connection with

the lytic-bearing colonies of *Bact. coli*. The round colony when fished to broth grew normally and the supernatant fluid had no lytic action.

These two types of colonies, one a normal colony, the other the lytic carrying colony were obtained with every culture against which the Olsen bacteriophage was active, dysentery as well as typhoid, and had not been described by d'Herelle at the date of our first communication, February 16, 1921 (Kuttner, 1921). If one of the normal colonies is restreaked, nothing but typical round typhoid colonies develop which apparently do not carry the lytic agent. If one of the irregular lytic bearing colonies is restreaked, both normal and irregular colonies are obtained. In one experiment a lytic colony and a normal colony obtained by plating a dissolved culture (Olsen bacteriophage acting on the homologous typhoid culture) was restreaked daily for 15 successive generations. In no case did the restreaking of a normal colony yield anything but normal colonies. The restreaking of the lytic colonies regularly produced both types: lytic colonies and normal colonies. The relative proportion of the two types varied, depending upon what part of the lytic colony had been touched with the platinum needle.

Twort in his original article states that the degenerative changes he described with micrococci characteristically started at the edge of the colony. In working with typhoid and coli cultures we have observed a great variation in the lytic colonies which appears to be a fairly definite characteristic of certain strains. Thus, if the Olsen bacteriophage acts on a Mt. Sinai strain of typhoid, the lytic action is most intense at the center of the colony. Similarly, with another bacteriophage (Newton) acting on a *Bact. coli* culture, lysis also begins at the center and has done so consistently with this strain. In restreaking a lytic colony, the greatest proportion of lytic colonies are obtained if the original lytic colony is touched at the point where the lytic process appears to be most active, whether this happens to be at the center of the colony or at the periphery. In our experience, in most cases, even if the lytic area of the colony was touched with platinum wire as delicately as possible, a certain

number of what appeared to be absolutely normal colonies developed. On the other hand, we do not agree with Bail's (1921) statement that after a few generations, the colonies carrying the lytic agent revert to normal. This depends as pointed out above, upon the manner of restreaking. Bordet and Ciuca report that the culture of *Bact. coli* derived from a lytic colony was still lytic after 150 transplants.

If a series of lytic typhoid or dysentery colonies are examined under the microscope, it will be found that there are often between the lytic colonies, minute transparent granular masses that have been called "appearances" by previous workers. They are structureless deposits which fail to grow when transplanted. It will also be found that the lytic colonies owe their irregular shape to the fact that their edges or centers, as the case may be, have faded into these "appearances." All variations between a fairly large lytic colony with only a little transparent material and the entirely translucent "appearances" which can only be seen with the microscope, occur. These transparent masses probably represent the débris left when a colony composed entirely of "sensitive" bacilli is dissolved and all the organisms killed.

"Susceptible" and "resistant" individuals in a single culture

Thus it is apparent that the lytic agent separates any susceptible culture into three types of individuals: The most susceptible bacillus whose descendants are all represented by the "appearance;" the susceptible bacillus whose descendants are both sensitive and resistant, represented by the lytic colony; and the bacillus which is itself resistant, and gives rise to nothing but resistant descendants, represented by the normal colony. These three types of individuals must preëxist in the culture as is indicated in the following experiment:

A young typhoid culture is washed off in salt solution and the emulsion is divided into three tubes and placed in a freezing mixture for thirty minutes. In the same way bacteriophage filtrates, undiluted and diluted 1:1000 are brought to a temperature of between 5° and

6°C., and just prevented from freezing solid. Some sterile salt solution and some centrifuge cups are also cooled. After half an hour, the undiluted bacteriophage is added to one tube of emulsion and the diluted bacteriophage to another, and the tubes are shaken. The ice salt solution is added to the control tube. The mixtures are kept in the freezing mixture and plated with the least possible loss of time. In both cases with the undiluted and diluted bacteriophage, "appearances," lytic colonies and normal colonies develop, whereas the salt control shows only normal colonies.

This experiment showed that the three types of bacilli pre-existed in the emulsion. The bacilli in the normal colony had been exposed to the same amount of lytic agent as the other organisms, and had apparently failed to unite or adsorb any bacteriophage, or if they had united or adsorbed the lytic agent, were not affected by it. The question that now arose was to find out the nature of the union between the susceptible bacilli and the lytic agent. Was it a definite union or merely an adsorption? The next step was also carried out at as low a temperature as possible, so as to prevent the growth of the bacilli.

The three tubes of the above experiment were placed in the cooled centrifuge tubes, and centrifuged to throw down the organisms. The supernatant fluid was poured off, the centrifuge cups and the tubes containing the sediment of bacteria recooled. Some of the cold salt solution was added and the sediment thoroughly emulsified by means of a capillary pipette which had been cooled. This process of washing the bacilli was repeated twice, and the emulsion then streaked.

The tube in which the bacilli had been in contact with concentrated bacteriophage yielded "appearances," lytic colonies and normal colonies. The tube to which the diluted bacteriophage had been added showed only normal colonies, as did the salt control.

Did the concentrated bacteriophage divide the culture into three types of bacilli after one single short contact, or was it a question of the concentrated lytic agent not having been completely removed by the washings? In the latter case it was the mechanical adhesion of the bacteriophage to the organisms

that had separated the culture into the three individuals of different degrees of susceptibility as they grew out in the agar streak. But in any case, it is possible to state that all susceptible cultures contain individuals of three different potentialities in respect to lytic agents. Whether the so-called resistant individuals that make up the normal colony represent, as has been claimed, the descendants of an individual that became immune to the small amount of bacteriophage that adhered to it, and has conferred this immunity to his descendants; or whether the physical surface of different bacteria vary so that the lytic agent adheres more readily to some individuals than to others, remains an open question.

Bacteriophage action, therefore, seems to depend on two main factors: the concentration of the lytic agent and the number of susceptible bacteria. This is well illustrated by the action of the lytic agent on cultures on solid media. If the lytic agent is added undiluted to an agar slant which is subsequently inoculated with a susceptible culture, practically no growth occurs. If high dilutions of the lytic agent are added, growth is normal except for certain transparent areas that occur at various points of the culture. The number and size of these transparent areas diminishes with the increasing dilution of the lytic principle. D'Herelle considers that each clear spot represents a colony of the virus. He believes that the concentration of the lytic agent at definite points in this way is characteristic of living things. Gratia's (1921) interpretation of these facts seems equally plausible at the present stage of our knowledge: each culture is made up of a whole scale of individuals ranging from the most susceptible to the most resistant. As the lytic agent is diluted, it is capable of dissolving only the most sensitive organisms which occur at various points of the culture.

It seemed of interest to determine whether the normal colonies that developed after lysis were totally and permanently refractive to further exposure to the lytic agent.

A normal resistant colony obtained by the action of the Olsen bacteriophage on strain 18 was isolated and to this culture the lytic agent

was again added in a dilution of 1:10. Lysis occurred more slowly than with a normal culture, but was definite. The tube was streaked and a resistant colony isolated and this second culture again exposed to the lytic agent. This was repeated four times and in the last case, definite clearing with the resistant culture did not take place, although on streaking the turbid culture a small number of lytic colonies were obtained. The resistant culture obtained after four successive exposures to the lytic agent was subcultured for five generations on agar and then again exposed to the lytic agent. It has lost a great part of its resistance and was nearly as susceptible as the stock culture.

Bordet and Ciuca (1921b) fished a lytic colony of *Bact. coli* to broth and kept it in the incubator for eight days and for twenty-two days at room temperature. At the end of this time they reisolated the culture and found it entirely resistant to lysis. They do not state, however, whether it again became susceptible on subculture. More recently, Eliava and Pozerski (1921) reported that they isolated a Shiga culture that had resisted lysis. It was resistant to bacteriophage action, but after 8 transplants it became more susceptible again.

Variations in typhoid strains—can a non-susceptible strain become susceptible?

The proportion of sensitive and resistant bacilli in a given culture could, therefore, be varied by exposure to a lytic agent. A culture which had become definitely resistant could be made susceptible again by transplantation. It seemed probable that certain strains of typhoid which were naturally resistant to lysis might be rendered susceptible by altering the cultural conditions under which they were growing. As stated above, the Rawlings strain of typhoid had consistently been resistant to lysis by the Olsen bacteriophage. This strain was known to ferment xylose slowly, whereas all the other strains that were susceptible to this particular lytic agent, fermented xylose rapidly.

It seemed, therefore, worthwhile to obtain a variant of Rawlings which fermented xylose rapidly, and see if it was more susceptible to lysis than the stock strain.

The Rawlings strain was streaked out on agar plates containing 1 per cent xylose according to the method described by Morishima for obtaining daughter colonies. The plate was sealed with plasticene and incubated four days. At the end of this time typical daughter colonies had developed which when fished to xylose broth, fermented this sugar in twenty-four hours, whereas the stock strain usually required seven days to ferment. The xylose rapid fermenting variant of Rawlings and the slow fermenting stock culture were now exposed to the Olsen bacteriophage, but no lysis was obtained and no lytic colonies developed on subculture.

Since Rawlings was a very old stock culture, it was thought possible that it had become resistant, due to the fact that it had been held as a stock culture and transplanted at long intervals from dried cultures. It was, therefore, transplanted daily for eight generations on moderately alkaline (pH 7.6) and very alkaline agar (pH 9); since it had been shown by Gratia that an alkaline reaction favors lysis, it was thought that possibly susceptible bacilli might develop as the result of growth in alkaline media. This did not prove to be the case and no evidence of lysis was obtained when these cultures were exposed to the Olsen bacteriophage. We have now, however, succeeded in obtaining a susceptible variant of the Rawlings strain. At the same time that the above experiments were being carried out, the stock Rawlings strain was transplanted to 100 cc. broth and allowed to remain in the incubator for four months and then left at room temperature for two months. The organism remained in the broth from June, 1921, until November, 1921. It was then isolated and identified as typhoid by fishing to Russell double sugar medium, on which it gave a typical reaction, and by the agglutination test. This strain reisolated from the broth proved susceptible to the Olsen bacteriophage and subsequent transplants from this culture have remained so for a period of three months.

Isolation of a resistant variant from an originally susceptible strain without exposure to bacteriophage

In April, 1921, Gratia reported that he had obtained a resistant variant from his susceptible *Bact. coli* culture by reisolating it

from the pellicle formed in an old broth culture. Pinhead, glassy colonies protruded from this pellicle, and by subculturing one of these, he obtained a resistant culture. The resistant culture was more motile and also more virulent than the stock culture. The resistance of this resistant variant was not, however, absolute. It was much more marked in acid than in alkaline media.

During May, 1921, it was suddenly noticed that the stock strain of typhoid, no. 18, which was transplanted almost daily, had become resistant to various agents (see below) which had formerly caused it to undergo lysis. It was then tested against the Olsen bacteriophage and it was found that no lysis was obtained with any of the transplants of no. 18 made after May 14. By going back to a culture of April 23, a susceptible transplant of no. 18 was again obtained. The sudden development of resistance on the part of this strain cannot be attributed to aging in this case, since the culture was in daily use. It was found that the stock agar we had been using for transplanting this culture had been somewhat more acid (pH 7) than we had used before, and we thought that perhaps the predominance of the resistant individuals over the susceptible might be attributed to this.

We, therefore, transplanted a susceptible and a resistant variant of no. 18 twice daily on acid, pH 6.8, agar and on alkaline pH 8.2 agar for twelve generations. We transplanted as rapidly as possible because we knew that the maximum number of susceptible individuals occurred in young cultures. We then tested these four different cultures (18 susceptible, twelfth generation, agar pH 6.8; 18 susceptible, twelfth generation, agar pH 8.2; 18 resistant, twelfth generation, agar pH 6.8; 18 resistant, twelfth generation, agar pH 8.2) against the Olsen bacteriophage.

The susceptible strain was still susceptible from both the acid and the alkaline medium, and the resistant strain still resistant from both media. At the same time, June, 1921, that the above experiment was done, the two variants of no. 18 were inoculated into 100 cc. of broth each, and allowed to age in the same way as the Rawlings culture described above.

In summing up our results with various strains of typhoid, we may state that we do not understand the factors that determine the susceptibility or resistance of a culture. The reaction of the medium and the rapidity of transplantation do not seem to affect the relation of susceptible and resistant individuals in a given culture. A culture that had been isolated for six months suddenly became resistant without being exposed to bacteriophage, and a different transplant of the same original culture has remained susceptible for a year and a half, and has been used constantly in various tests. In the case of the Rawlings culture, originally resistant, a susceptible variant has been obtained by reisolating the strain from an old broth culture. In conclusion, we may say that in typhoid cultures the relation between susceptible and resistant organisms is not a very stable one, and that for reasons of which we are ignorant, one of the other type may predominate.

II. ISOLATION OF THE LYTIC AGENT FROM THE TISSUES AND BLOOD OF NORMAL ANIMALS

The action of the Olsen bacteriophage on typhoid and dysentery cultures was studied in detail in order to familiarize ourselves with the lytic process. It was fairly obvious, however, that unless one was willing to accept d'Herelle's theory of a living virus, no progress in finding out the nature of the phenomena could be made, unless it was possible to isolate a lytic principle without the interaction of the living animal body. The fact that the lytic agent could be transmitted indefinitely in series and was active only against vigorously growing bacilli, suggested that the bacteriophage principle might be derived from the organisms themselves.

We proceeded on a theory first advanced by d'Herelle, but discarded by him in favor of the living virus. According to this theory, bacteriophage action was due either to the activation of the natural autolysin contained in all bacteria, or to the removal of an autolysin-inhibiting substance. Once this natural autolysin was liberated it could in turn liberate more of the autol-

ysin from the next generation of bacteria and so on indefinitely. This hypothesis will be again discussed at the end of the paper.

The work of Cantacuzene and Marie (1919) on the action of intestinal mucosa on cholera vibrios and that of Turro (1921) on the action of tissue extracts on a variety of bacteria, suggested that a bacteriophage principle might have played some part in the experiments reported by these workers.

With the work of Cantacuzene and Marie, and Turro in mind, we prepared various tissue extracts as has been already reported in a brief note.

Bacteriophage action started with extracts of intestinal mucosa

Experiment. March 18 the small intestine, large intestine, and a part of the abdominal wall of 3 normal guinea-pigs which had been bled to death for complement, were removed. The tissues were thoroughly washed in running water and cut into small pieces with a scissors. Without any preliminary drying each of the three types of tissue was divided roughly into 4 parts and placed in 12 bottles. Each type of tissue was extracted in 50 per cent glycerol, 25 per cent glycerol, 5 per cent glycerol and salt solution, 50 cc. of fluid being added to each bottle. The bottles were put on ice from March 18 until March 21 and then plated to see to what extent they were contaminated. The tissue to which the 50 per cent glycerol had been added showed no growth. The plates from the other bottles developed a small number of colonies, mainly staphylococci.

All the bottles were then incubated from March 22 to March 29. On March 29 a small amount of the supernatant fluid from the bottles of small intestine, large intestine and abdominal wall extracting in 50 per cent glycerol were centrifuged and filtered through a Berkefeld filter to obtain a clear fluid. These filtrates were titrated to make sure that the reaction was neither sufficiently acid nor alkaline to interfere with the growth of the typhoid bacillus. The filtrates were tested in the following way against the typhoid strain 18, 0.1 cc. of a fairly heavy saline emulsion from one eighteen hour agar slant being added to 2 cc. extract broth, pH 7.8. The amount of emulsion added was sufficient to make the tubes slightly but definitely turbid.

March 30

TUBE	AMOUNT OF BROTH	CUL- TURE 18	PREPARATIONS TESTED	4 HOURS	24 HOURS	
	cc.	cc.				
1	2	0.5	0.5 cc. 50 per cent glycerol, small intestine, pH 6.8	++	+1	Normal growth
2	2	0.5	0.25 cc. 50 per cent glycerol, small intestine, pH 6.8	++	+	Few lytic colo- nies
3	2	0.5	0.50 cc. 50 per cent glycerol, large intestine, pH 7.0	++	++	Normal growth
4	2	0.5	0.25 cc. 50 per cent glycerol, large intestine, pH 7.0	++	++	Normal growth
5	2	0.5	0.50 cc. 50 per cent glycerol, abdominal wall, pH 6.8	++	+	Normal growth
6	2	0.5	0.25 cc. 50 per cent glycerol, abdominal wall, pH 6.8	++	+1	Normal growth
7	2	0.5	0.50 cc. 50 per cent glycerol, solution	++	++	Normal growth
8	2	0.5	0.25 cc. 50 per cent glycerol, solution	++	++	Normal growth
9	2	0.5	0.50 cc., Olsen bacterio- phage	±	±	Normal growth
10	2	0.5	0.25 cc., Olsen bacterio- phage	±	±	Lytic colonies
11	2	0.5	0.50 cc., salt solution	++	++	Normal growth
12	2	0.5	0.25 cc., salt solution	++	++	Normal growth

++ = turbidity equal to control.

- = complete clearing.

The tubes were placed in the incubator for four hours and examined, and then put back in the incubator for twenty hours more, and again examined for clearing. The tubes were all plated at this time and then heated at 56°C. for thirty minutes to prevent the overgrowth of resistant types.

The twenty-four hour plate of tube 2 showed lytic colonies identical with those of tube 10, the lytic control tube. The platings from none of the other tubes showed any lytic colonies. Tube 1 where a greater amount of active intestinal extract was used showed no lytic colonies, but this is often the case, since tubes to which a greater amount of the lytic agent whatever its origin is added, usually clear faster and are then often more rapidly overgrown with the resistant organisms. The same thing occurred in this particular experiment in the control tube 9. Glycerol alone failed to give rise to lytic colonies. The degree of clearing is a very unreliable criterion, especially on first isolation of a lytic

agent. The development of lytic colonies is the most definite proof of the presence of a lytic agent. Clearing alone is never sufficient evidence for bacteriophage action.

The lytic colonies obtained from tube 2 were studied in detail and found to have all the characteristics of lytic colonies obtained by action of the Olsen bacteriophage.

April (1) The supernatant fluid of the bottle with the small intestine extracting in 25 per cent glycerol was centrifuged and filtered through a Berkefeld. Another lot of the bottle with small intestine and 50 per cent glycerol was filtered, and then these filtrates small intestine 25 per cent glycerol incubating March 22 to April 1, small intestine 50 per cent glycerol incubating March 22 to April 1, together with the first extract found active small intestine March 22 to March 29 (tested March 30) were set up on April 1. Tube 2 in the previous protocol was also tested to see whether the lytic agent isolated from the mucosa was transmissible in series. The test was set up in the same number as the previous one.

April 1

TUBE	AMOUNT OF BROTH	CUL- TURE 18	PREPARATIONS TESTED	PLATES AFTER TWENTY-FOUR HOURS
	cc.	cc.		
1	2	0.5	0.50 cc. 50 per cent glycerol, small intestine, March 22 to March 29	Normal growth
2	2	0.5	0.25 cc. 50 per cent glycerol, small intestine, March 22 to March 29	Many lytic colonies
3	2	0.5	0.50 cc. 50 per cent glycerol, small intestine, March 22 to April 1	Normal growth
4	2	0.5	0.25 cc. 50 per cent glycerol, small intestine, March 22 to April 1	Many lytic colonies
5	2	0.5	0.50 cc. 25 per cent glycerol, small intestine, March 22 to April 1	Normal growth
6	2	0.5	0.25 cc. 25 per cent glycerol, small intestine, March 22 to April 1	Many lytic colonies
7	2	0.5	0.50 cc. tube 2, March 30	Many lytic colonies
8	2	0.5	0.25 cc. tube 2, March 30	Many lytic colonies
9	2	0.5	0.50 cc. Olsen bacteriophage	Many lytic colonies
10	2	0.5	0.50 cc. salt	Normal growth

The 25 per cent glycerol extraction of the pooled small intestine tissue was also found active; the result obtained with the 50 per cent glycerol on March 30 was repeated. The lytic agent derived from the mucosa was found to be active in the second generation (tubes 7 and 8).

The lytic process started with these extracts in no way differed from the bacteriophage action started with Olsen bacteriophage. It could be transmitted in series and, the speed of lysis and number of lytic colonies obtained increased markedly in the first three or four generations. Extracts of the large intestine with 50 per cent glycerol also proved active after a longer period of extraction March 22 to April 25. Extracts of the abdominal wall were never found to be lytic. The preparations with 50 per cent glycerol and salt in spite of the addition of 5 per cent chloroform, became so contaminated that they were discarded without being tested.

It was thus shown that lysis transmittable in series could be started with glycerol extracts of large and small guinea-pig intestinal mucosa in certain instances. We also tried to see if this property was confined to the intestinal mucous membrane, or whether it could also be found in other organs. We, therefore, prepared a liver extract exactly according to the directions of Turro (1921).

Bacteriophage action started with an extract of liver tissue

March 31, 1921, a liver taken from a normal guinea pig was minced, shaken up in about 6 volumes of acetone, dried in vacuo and pulverized. To approximately 1 gram of liver powder, 20 cc. of sterile salt solution were added. To one tube 40 drops of chloroform were added, to the other a small amount of dry sodium fluoride. Both tubes were incubated fifteen hours. At the end of this time the tube to which the sodium fluoride had been added was contaminated with a large Gram positive bacillus. The tube to which the chloroform had been added was apparently sterile. The former was centrifuged and filtered through a Berkefeld, the other was merely centrifuged. Both these preparations were tested against typhoid no. 18 in the same way as the intestinal extracts. The liver extract to which the chloroform had been added was not as active in the first generation as the one to which sodium fluoride had been added. The following protocol where the specificity of the liver extract is tested is of interest.

The liver extract (sodium fluoride March 31) was tested against the following 6 strains: typhoid no. 18, typhoid Rawlings, Shiga dysentery, Mt. Desert, cholera and the *Bact. coli* obtained from Dr. Bordet. In this case, the extract was tested against each culture in two ways by lysis and by inhibition; that is, in the former case the 2 cc. of broth, the amount used consistently throughout these tests, was inoculated with a sufficient amount of the culture emulsion taken from an eighteen hours slant to produce definite clouding. In the latter case the tube was merely heavily inoculated without producing visible turbidity. This method of setting up the tests in two ways is to be recommended where a new and feeble lytic agent is being tested, because the danger of overgrowth with resistant types always exists. By inoculating a large and small number of organisms, the chances of finding lytic colonies or transparent areas, which is the surest indication of bacteriophage action, at one or the other intervals of plating, is increased. In the majority of our more recent tests we have adopted the following routine: The tubes are plated after two hours and then left standing at room temperature, and then plated again.

April 12, 1921

TUBE	AMOUNT OF BROTH	CULTURE		LIVER (NaFl) EXTRACT APRIL 1	PLATED AFTER FOUR HOURS
	cc.			cc.	
1	2	Typhoid no. 18	0.50 cc.	0.2	Few lytic colonies
2	2	Typhoid	0.05 cc.	0.2	Little growth, few lytic colonies
3	2	Rawlings	0.50 cc.	0.2	Normal growth
4	2	Rawlings	0.05 cc.	0.2	Normal growth
5	2	Shiga	0.50 cc.	0.2	Transparent areas
6	2	Shiga	0.05 cc.	0.2	Very delicate growth, no lytic colonies
7	2	Mt. Desert	0.50 cc.	0.2	Normal growth
8	2	Mt. Desert	0.05 cc.	0.2	Transparent areas
9	2	Cholera	0.50 cc.	0.2	Normal growth
10	●2	Cholera	0.05 cc.	0.2	Normal growth
11	2	Bordet <i>B. coli</i>		0.2	Normal growth
12	2	Bordet <i>B. coli</i>		0.2	Normal growth

A similar series of tubes was set up at the same time in which sterile salt solution was substituted for the liver extract. The plates from these tubes all showed normal growth. The action of sodium fluoride alone was also tried, and in those concentrations which did not interfere with growth, normal colonies were obtained. It is interesting to note that the Rawling strain is resistant to the liver extract in the same way that it is to the Olsen bacteriophage. The range of activity of this liver extract was about the same as that of the Olsen bacteriophage. Cholera and the Bordet *Bact. coli* were not affected by either.

The lytic process started with the liver extract was shown to be transmissible in series. In order to demonstrate even more clearly that the same substance was at work in the liver extract and in the Olsen bacteriophage, 2 rabbits were immunized, one with dissolved typhoid culture filtrate (Olsen bacteriophage), the other with the active liver extract. The rabbits were injected intravenously at intervals of four or five days. The rabbit 1098 injected with the Olsen dissolved culture filtrate, was bled the 4th day after the sixth injection. The rabbit 335 injected with active liver extract, was bled one day after the fourth injection.

The normal and immune sera of these two rabbits were then tested as to their ability to convert lytic colonies to normal by the method described by Bordet (1921c).

Experiment. June 13, 1921, 4 agar slants were inoculated in the following way:

- (1) with 7 drops of the normal serum of rabbit 1098.
- (2) with 7 drops of the immune serum of rabbit 1098.
- (3) with 7 drops of the normal serum of rabbit 335.
- (4) with 7 drops of the immune serum of rabbit 335.

These tubes were then incubated in the inclined position overnight. On the following day they were found to be sterile. They were then inoculated with equal amounts of a broth fishing of a lytic colony obtained by the action of the Olsen bacteriophage on no. 18.

(5) A plain agar slant was also inoculated with the same material. After eighteen hours the following result was obtained:

Tube 1 showed a small number of lytic and normal colonies.

Tube 2 showed heavy confluent growth.

Tube 3 showed a small number of lytic and normal colonies.

Tube 4 showed a fair amount of confluent growth, no lytic colonies.

Tube 5 showed a small number of lytic and normal colonies.

This experiment was repeated two days later, and the same result was obtained. The antilytic serum produced by injecting the active extract (liver) had the same power to neutralize the growth of lytic colonies as the antilytic serum produced by the inoculation of Olsen bacteriophage. The normal serum of neither of the rabbits interfered with the development of the lytic colonies.

Bacteriophage action started with normal rabbit serum

Bordet in describing the production of antilytic sera, mentioned the fact that the lytic agent persisted in the circulation for a period of forty-eight hours. In starting to immunize 2 rabbits, one with Olsen dissolved culture filtrate no. 1052, the other with active liver extract no. 335, we took normal bleedings and also bled again eighteen hours after the first injection to see whether the lytic agent could still be demonstrated in the circulation. We then tested the four bleedings obtained in this way as follows:

TUBE	AMOUNT OF BROTH	CUL- TURE 18	SERA TESTED	SIX HOUR PLATES
	cc.	cc.		
1	2	0.3	0.2 cc. normal rabbit serum no. 335	No lytic colonies
2	2	0.3	0.2 cc. rabbit serum no. 335 after first injection	Some lytic colonies and transparent areas
3	2	0.3	0.2 cc. normal rabbit serum no. 1052	Some lytic colonies
4	2	0.3	0.2 cc. rabbit serum no. 1052 after first injection	No lytic colonies
5	2	0.3	0.2 cc. salt solution	No lytic colonies

It was thus found that normal rabbit serum in some instances contained a lytic agent. Transmission of the lytic agent in series from tube 3 offered no difficulties. This experiment was repeated three times. The suspicion that the two tubes of no. 1052 had been accidentally mixed could be definitely eliminated because, in taking the normal bleeding, a large quantity of blood had been taken, since the normal serum would be required as

a control for future tests, whereas, only a small amount of blood was necessary for the demonstration of the persistence of the lytic agent in the circulation. It is also to be noted that the serum of the rabbit 335 injected with one dose of active liver extract, was active after eighteen hours.

During the past year out of 50 normal rabbits bled, the serum of 6 contained a lytic agent active against typhoid bacilli. No data were obtained as to what determined the presence of the bacteriophage principle in the circulation of these particular animals. The serum of 2 of these rabbits when bled again, failed to show any lytic activity, indicating that the condition was transitory. An active serum remained so for an indefinite period when stored on ice. The lytic agent in the serum was not related to the complement since the activity was not decreased by inactivation at 56° for thirty minutes, and in two instances seemed definitely increased after inactivation. The activity of two immune sera, one rabbit and one horse, was also tested, but they were found negative.

Possible connection between bactericidal titer of normal rabbit sera and bacteriophage action

It is of interest in this connection to recall the observations of Teague and McWilliams (1917) who found a great variation in the bactericidal titer of different rabbit sera, and observed that the bactericidal titer of immune rabbit serum was much lower than that of normal rabbit serum. It seemed possible that the explanation of the exceptionally high bactericidal titer of certain rabbits might be attributed to bacteriophage action, but had this been the case, it would have been extraordinary if the extremely characteristic lytic colonies had not been observed before. We consequently duplicated as closely as possible the tests made by Teague and McWilliams with one of our lytic sera. We were short of active serum and could use 0.7 cc. only instead of 1 cc. Seven tenths cubic centimeter of serum was inoculated with a fairly light suspension of typhoid bacilli and plated after five hours incubation and again after eighteen hours.

In both five-hour and eighteen-hour plates a small number of lytic colonies were obtained. We did not count the typhoid suspension accurately, and it is quite possible that with the use of such a large amount of serum and inoculating a relatively small dose of bacteria, only the resistant colonies might develop, giving a small number of perfectly typical colonies. Teague and McWilliams used streak plates in the same way that we do in determining lytic action. It is customary in most bactericidal tests to make "pour" plates, in which the recognition of a small number of lytic colonies would be extremely difficult. Whether or not bacteriophage action played a part in the results obtained by previous workers cannot be definitely stated, but in the future any one working on the bactericidal titer of normal rabbit sera will have to test for bacteriophage action. The differentiation of bactericidal action and lytic action can easily be made because of the heat resistance of the lytic agent. A small number of normal guinea-pig sera were also tested against typhoid, but none of them showed any lytic activity. A few isolated experiments with normal human sera were negative.

The lytic agents found in serum, like those described from other sources, were resistant to drying. One of the active sera which had dried completely in the bottom of a tube, when re-dissolved in a little salt solution, still proved active. We found that we were unable to absorb out the lytic agent from a serum by a single absorption with typhoid bacilli at 0°C.

Absorption of lytic agent from serum

One cubic centimeter of active rabbit serum no. 1052 and the saline emulsion of a fresh agar slant of the Mt. Sinai typhoid strain were both placed in a freezing mixture for thirty minutes. The serum and the bacteria were then combined and kept at a freezing temperature for four hours. The experiment was carried out at the low temperature to prevent the growth of the bacteria since we know that the amount of the lytic agent is increased with the growth of the organisms. At the end of this time, the mixture was centrifuged to throw down the organisms. The serum was pipetted off and heated at 58° for thirty minutes to kill any remaining bacteria. In order to prove that some of the lytic agent

had actually united with the bacteria, the organisms were washed three times (to eliminate the serum which had merely adhered) and then streaked out on an agar plate. At the same time, 1 cc. of sterile broth was added and the organisms incubated overnight, to see if the growth thus obtained would be normal. On the following day, this tube was also plated.

The plate made immediately after washing the bacteria showed no lytic colonies, but the plate from the broth tube showed definite evidence of lysis. The serum after being tested for sterility, was set up with the Mt. Sinai culture to see if the lytic agent had been absorbed out. Typical lytic colonies were obtained after the serum and culture had been in contact for three hours. Thus, four hours absorption was not sufficient to remove the lytic agent from the active normal rabbit serum.

The specificity of one of the active sera was tried out in one experiment. We were always very much handicapped by not being able to get a very large quantity of active normal serum, since we had no way of judging when a serum would be active. In this instance, the serum which was lytic for typhoid strain no. 18, was not active for Mt. Desert, but too little work has been done on this point to draw any conclusions.

Is the presence of the lytic agent in the circulation secondary to its presence in the intestinal canal?

At the same time that most of these experiments were carried out, d'Herelle's findings on the occurrence of bacteriophage in the feces of various normal animals were not known to us, so that no attempt was made to correlate the active rabbit sera with the presence of bacteriophage in the feces. In his book, d'Herelle states that he examined the feces of 2 rabbits and found that the filtrate obtained was active in one case against Shiga, Mt. Desert and Flexner dysentery bacilli, while the filtrate from the other rabbit showed only a very slight activity against the Shiga bacillus. When we first discovered the lytic agent in normal rabbit sera, we thought that the lytic activity could be attributed to some ferment-like activity which activated the bacteria to autolyze, since normal rabbits do not in any way come in contact with members of the typhoid-dysentery group.

But if a Shiga bacteriophage can be isolated from the feces of a normal rabbit, it may be argued that the sera of certain rabbits which happen to contain a bacteriophage against typhoid or Shiga in their intestinal tract, may be active because under certain circumstances the bacteriophage penetrates into the circulation from the intestine. Since normal rabbits do not have either typhoid or Shiga bacilli in their feces, it may be assumed that feces of these animals must contain organisms closely related to these bacteria in their fecal flora, since the bacteriophage is only known to exist in the presence of susceptible bacilli.

We devised the following experiment in our attempt to obtain some information on this point.

We bled 6 normal rabbits, and at the same time plated the feces of each on Endo plates. The fecal flora of rabbits does not seem to be very varied, since, in most cases, only two types of organisms were obtained, and in some, only one type. The plates were examined carefully under the microscope to see if any small lytic colonies of organisms more or less completely dissolved, could be detected in the direct plates, but none were found. The organisms obtained were isolated on Russell's medium. The fecal emulsion was incubated overnight and then heated at 60° for one hour, since we planned to test the fecal suspension for evidence of bacteriophage if any of the sera proved active. The sera were tested against the homologous strains or strain of *Bact. coli*, and also against other organisms. The protocol of only one rabbit serum will be given to show the method, since the experiment was entirely negative.

Rabbit serum no. 530 inactivated 56° for thirty minutes

TUBE	AMOUNT OF BROTH	CULTURE	SERUM	TWO HOUR PLATES	EIGHTEEN HOUR PLATES
	cc.		cc.		
1	2	No. 530 homologous <i>Bact. coli</i> (1)	0.3	No lytic colonies	No lytic colonies
2	2	No. 530 homologous <i>Bact. coli</i> (2)	0.3		
3	2	Typhoid no. 18	0.3		
4	2	Typhoid Mt. Sinai	0.3		
5	2	Typhoid Mallon	0.3		
6	2	Mt. Desert	0.3		
7	2	Newton <i>Bact. coli</i>	0.3		

The sera were thus tried against 7 different strains, 2 of the organisms being derived from the same rabbit's intestinal tract, but none of them proved active against either the homologous *Bact. coli* or the other cultures tried. Most of the workers who have studied stool filtrates from normal as well as pathological human cases, state that bacteriophage action could probably be demonstrated in every stool if it were tested against the right organisms. But the problem of finding the right organisms is not an easy one. This line of research has not been pursued further because of the discovery made by several different workers, including ourselves, that the bacteriophage principle can be obtained from the bacteria alone under certain circumstances without the action of any external agent.

We have thus been able to start lytic action with 2 different types of tissues derived from guinea-pigs, and also with normal rabbit sera. The intestinal mucosa of certain guinea-pigs extracted with glycerol when added to normal typhoid cultures produced lysis transmittable in series. In the same way liver extracts from guinea-pigs can in certain instances produce the same result. The presence of lytic agents in these tissues active against typhoid bacilli is, however, extremely rare. Normal rabbit sera (6 out of 50) also occasionally contain the lytic agent for typhoid bacilli, but we do not understand the conditions that determine the lytic activity of the blood of these animals. The experiments in which the bacteriophage action of these normal tissues or normal sera was shown, were always carefully controlled and the culture without the addition of the particular extract or serum, never gave any evidence of lysis.

III. EXPERIMENTS ON THE ORIGIN OF THE LYTIC AGENT IN THE BACTERIA THEMSELVES

Bacteriophage action may thus be started by a wide variety of agents from normal as well as from diseased animals, but whatever agent starts the process, it is clear that eventually in a series, the lytic agent must be derived from the bacteria themselves, unless we are willing to accept the parasitic nature of the lytic agent.

If the lytic agent is really an activated autolysin, it ought to be possible to isolate it from old, spontaneously autolyzing cultures.

On June 13, 1921, two bottles of 100 cc. of broth were inoculated respectively with the strain of the stock typhoid strain of typhoid no. 18 which had spontaneously become resistant to bacteriophage action and with the susceptible variant of no. 18. These broth cultures had remained in the incubator for a period of four months and had then been left at room temperature for two more months. On November 9, some of the supernatant fluid from these two bottles was removed, centrifuged and heated at 58°C. for thirty minutes. These heated supernatants were then tested against the susceptible variant of no. 18, which had been in constant use as a test culture. Large amounts were added because it was thought that if present at all, the lytic agent would be feeble. The test was set up as follows: 1 cc. amounts of the heated supernatants were inoculated directly with 0.1 cc. of no. 18, and at the same time, the test was carried out in the usual way, 2 cc. of broth being inoculated with 0.1 cc. of culture, and then 0.5 cc. of the material to be tested, added.

TUBE	AMOUNT OF BROTH	CUL- TURE 18	OLD BROTH CULTURES TESTED	FIVE HOUR PLATES
	cc.	cc.		
1	2	0.1	1.0 cc. old broth 18D (resistant variant)	Transparent areas Lytic colonies
2		0.1	0.5 cc. old broth 18D (resistant variant)	
3	2	0.1	1.0 cc. old broth 18 (susceptible variant)	Normal growth
4		0.1	0.5 cc. old broth 18 (susceptible variant)	Normal growth
5	2	0.1	1.0 cc. sterile broth	Normal growth
6		0.1	0.5 cc. salt	Normal growth

This test was repeated using the filtrate of this old broth culture, together with the previously used heated supernatant fluid. The same result was obtained. The strain of typhoid was then reisolated from this culture. Only normal colonies were obtained on streaking. A fresh agar slant made by transplanting one of these colonies was tested against the Olsen bacteriophage. It was still wholly resistant.

Thus a bacteriophage, active against the susceptible variant of no. 18, had been extracted after prolonged incubation from the resistant variant. Under the heading, variations in typhoid strains, above, it will be noted that this strain had become resistant to lytic action without exposure to the lytic agent. The streak from the bottle of 18D showed that it had become contaminated with a *Staphylococcus albus* and a diphtheroid and it was thought that possibly the question of symbiosis might play a part in the production of bacteriophage from the organisms themselves. Carrère and Lisbon (1922) have recently had a similar idea, but this has now definitely been disproved, since pure cultures yield bacteriophage.

At the time we obtained the above result, we had not read Bail's (1921) article of September 15, 1921, in which he stated that he had isolated a bacteriophage from 3 old broth cultures active against Flexner dysentery bacilli. He does not state what organism was originally inoculated into the old broth. In a more recent article, Otto and Munter (1921) have succeeded in isolating bacteriophage in 9 instances, active against several organisms of the typhoid-dysentery group, obtained from old broth cultures of Flexner, Mt. Desert and Shiga dysentery and typhoid bacilli, respectively. The broths varied from three weeks old to six months. Unfortunately, the authors do not state whether they reisolated the strain from the broth to see whether it was resistant to the bacteriophage produced in the fluid in which it was growing, nor in case the bacteriophage was obtained in an old typhoid culture, for instance, whether the lytic fluid was active against the homologous typhoid strain, or only against other typhoid strains. It would also be of interest to know whether the broth was inoculated with old stock cultures, or recently isolated ones.

We have succeeded in obtaining another bacteriophage from a two months old typhoid culture (no. 18 susceptible) active against Shiga dysentery, but apparently against no other organism. We have not tried to date, to see whether by allowing it to act on Shiga dysentery for a few generations, we could obtain a bacteriophage active against typhoid. Culture no. 18 has been isolated for a year and a half.

The most interesting results in regard to obtaining bacteriophage directly from the bacteria themselves have been obtained by Callow in this laboratory, and are about to be published. She has found that the filtrates of certain strains of staphylococci prepared as described below, added to young broth cultures of certain other strains of staphylococci, proved lytic.

The filtrates were obtained by growing staphylococci on agar plates for eighteen hours, washing off the growth in sterile broth, or better, in distilled water containing 0.02 per cent sodium hydroxide, shaking the emulsion for thirty minutes to an hour, and then filtering through a Berkefeld filter. The filtrate thus obtained was active for 1 or more strains of staphylococci, but in no instance against the homologous strain. It was thus possible to wash the lytic agent directly off certain strains of staphylococci from comparatively young cultures active against other strains. Miss Callow has obtained the same results with filtrates of young broth cultures, but the results have been much less constant than with the washings from the agar cultures. In the broth cultures also the filtrate was only in very rare instances, active against the homologous strain originally inoculated into the broth, and this result was only obtained with older broth cultures. The strains used by Miss Callow in these experiments had been isolated from boils for a period of three or four months and had been transplanted frequently. Miss Callow had not tried the method of washing off agar growths with older stock cultures of staphylococci, so that we cannot state that old as well as recently isolated strains produce a bacteriophage principle by this method.

A small number of experiments have been done using the method originated by Miss Callow with strains of typhoid and dysentery bacilli, but to date without success. A variety of strains were tried. No. 18 susceptible and 18D, the resistant variant, were used, also the Shiga bacillus, which is generally agreed to be the most susceptible organism of the typhoid-colon-dysentery group. Another recently isolated strain of typhoid and a susceptible *Bact. coli* culture were also tried. Six hour, twenty-four hour and four-day agar growths were shaken in alkaline solution, but none of the filtrates tested against a variety of cultures showed any lytic action. However, this work has been done very recently and most of the strains only tried once so that the results are not wholly conclusive.

Summary of isolation of bacteriophage from bacteria themselves

It can be stated that in the hands of four different workers, Bail, Otto and Munter, Callow and ourselves, it has been possible to isolate a bacteriophage from the bacteria themselves. With the typhoid-dysentery-colon group, this can most easily be done with old broth cultures. With staphylococci, the best results are obtained by washing off young agar cultures. The bacteriophages thus obtained are in the results obtained by Callow and ourselves, not usually active against the strain with which the broth or agar was originally inoculated. In the case of the bacteriophage, active against Shiga bacilli, obtained from an old typhoid culture, the typhoid culture had been isolated for a period of over a year, and been constantly used as a control and never given any signs of spontaneous lysis.

A recent paper by Lisbon and Carrère (1922) is of interest in this connection.

These authors state that they have been able to obtain a bacteriophage active against Shiga dysentery by inoculating a Shiga broth culture with a recently isolated *Bact. coli*. After incubating this mixed culture for a variable length of time, the broth is filtered. This filtrate is then added to a culture of Shiga bacilli and carried along for three or four generations. A bacteriophage active against the Shiga bacilli is finally obtained. They obtained a similar result by using a strain of *Proteus*, but they do not state whether it was recently isolated or not.

D'Herelle (1922) has answered Lisbon and Carrière by saying that since the strains of *Bact. coli* that they used were all recently isolated from stools and urine, the probability is that they were dealing with organisms that were carrying a bacteriophage, although apparently no evidence of lysis in the *Bact. coli* culture was observed. D'Herelle has described in his book (page 58) what he calls mixed cultures of bacteria and the ultramicroscopic virus in which the resistance of the bacteria is sufficient to prevent the formation of lytic colonies, and the virus is carried by what appear to be perfectly normal colonies.

Bordet and Ciuca were the first workers to show that the lytic agent could be carried with a certain type of *Bact. coli* colony ob-

tained after the culture was exposed to lysis. We have found that in the case of typhoid and dysentery cultures, bacteriophage action always divided the culture into lytic-bearing and what appeared to be non-lytic normal colonies. We streaked one of these normal colonies for fifteen successive generations, as reported above, on agar without ever obtaining anything but normal colonies. We also fished some of these normal colonies to broth and tried out the supernatant fluid comparison with the supernatant fluid of broth fishings of lytic colonies, without obtaining any evidence that the lytic agent was carried by these normal colonies. We, therefore, concluded that only the colonies in which we could definitely see evidence of lysis—i.e., lytic colonies—carried the virus.

In obtaining bacteriophage principles from old broth cultures and from agar washings of young growths of *Staphylococcus* (Callow) it seemed at first that we had definitely proved that the lytic material was derived from the bacteria themselves. But we realize now that the argument will not be conclusive until we are able to demonstrate lytic activity in old broth cultures of old laboratory strains, or perhaps by the aid of the Barber single cell isolation method. In both instances where we obtained lytic activity with old broth cultures, the culture used had in one instance been isolated six months and in the other over one year, but it was originally derived from a typhoid case in which the feces were shown to contain a potent bacteriophage. We have also described in detail how this culture fluctuated in resistance to the Olsen bacteriophage. The defenders of the filtrable virus theory would see proof in this for their claim that an apparently normal culture can carry a bacteriophage without giving any evidence of it, since this culture six months after isolation suddenly became resistant and when inoculated into broth, after prolonged incubation, produced a bacteriophage. The sudden resistance of this strain might, however, also be explained more simply by saying that in the course of the transplantation a series of resistant bacilli, which we know to exist in every culture, happened to be transferred for successive transplants until nothing but resistant bacilli were present.

The German writers do not give the history of the strains used by them. Miss Callow to date has only been successful with strains of recent isolation. We cannot, therefore, at the present time, completely exclude the possibility that apparently normal bacteria may carry an ultramicroscopic parasite.

IV. RESISTANCE OF THE LYTIC AGENT

Acetone resistance (Olsen bacteriophage)

A great many workers have tried to disprove the living virus theory in regard to bacteriophage action, notably Kabeshima, by pointing out the great resistance of the lytic agent. In our first attempt we were unable to confirm the method devised by Kabeshima for isolating the lytic material by precipitation with acetone. Recently, however, we have been able to demonstrate lytic activity in an acetone precipitate. Instead of starting with the simple dissolved culture filtrate obtained by the action of the Olsen bacteriophage on a typhoid culture, we concentrated the filtrate to one-tenth its volume by evaporation with a fan.

To 50 cc. of this concentrated lytic broth 150 cc. of acetone were added and the mixture was allowed to stand at room temperature and shaken from time to time. At the end of 48 hours the acetone was evaporated off until only a syrupy brown liquid remained. This was centrifuged, and a small amount of yellow precipitate obtained. The supernatant fluid was pipetted off and the precipitate partially redissolved in salt solution. Both the salt solution solute thus obtained and the supernatant fluid proved active.

It may be argued that the only reason that this concentrated lytic broth was able to withstand the forty-eight-hour exposure to acetone was because it was protected by the concentration of the proteins in the broth; but in any case, it shows a fairly high degree of resistance for the lytic agent. We concentrated the lytic precipitate in the first place to see if we could obtain a lytic agent of much greater potency. In one experiment the lytic titer was very much increased after concentration, but in subsequent tests the increase in titer was not so striking. The

Olsen bacteriophage is active in a dilution of 1:1,100,000, with most batches, without being concentrated.

We also precipitated 1 volume of the concentrated lytic broth with 9 volumes of acetone, and centrifuged the emulsion thus obtained, and redissolved the precipitate in salt solution. The precipitate dissolved very readily. The solution thus obtained, however, showed no lytic activity.

Typhoid dissolved culture filtrate (Olsen bacteriophage), if concentrated at one-tenth its volume, will resist exposure to 3 volumes of acetone for forty-eight hours. If, however, exposed to 9 volumes of acetone for a very short period, it is destroyed.

Resistance to alcohol precipitation

D'Herelle, in his book, states that by means of alcohol precipitation he has been able to separate the filtrable virus from the enzyme by which it acts.

If the lytic filtrate is exposed to 95 per cent alcohol for forty-eight hours the virus is destroyed, but the enzyme by which the virus does its work is still active. If, therefore, the redissolved precipitate obtained by adding 9 volumes of alcohol to one of lytic broth, is added to a turbid broth culture of a susceptible organism the clarification of the broth takes place, but the lytic agent is no longer transmissible in series, and no lytic colonies are obtained on streaking the dissolved cultures. The virus according to d'Herelle resists exposure shorter than forty-eight hours.

We have not to date been able to verify this particular experiment, but we have confirmed the extreme resistance of the lytic agent to alcohol precipitation. Exposure of 1 volume of concentrated lytic broth to 12 volumes of absolute alcohol for two hours did not destroy its activity. Miss Callow, using saline washings of agar cultures which contain a minimum of protective protein, has noted resistance to precipitation with alcohol for twenty-four hours.

The lytic agent, therefore, is more resistant than any form of life with which we are familiar, with exception of certain spores and an ultramicrobe parasitic on tobacco cited by d'Herelle.

We have also verified the observation of other workers that the lytic agent is resistant to exposure to 50 per cent glycerol and chloroform. It is interesting to note that Rettger in 1905 had found that autolysins of the bacteria were more resistant to 10 per cent chloroform than to 10 per cent toluol. We exposed the Olsen bacteriophage to 10 per cent chloroform and 50 per cent glycerol for a period of thirteen days without loss of activity.

V. IS THE LYTIC AGENT ANTIGENIC?

We have immunized 4 rabbits with typhoid cultures dissolved by the action of the Olsen bacteriophage. All the rabbits were injected intravenously and stood the injections well. One rabbit received altogether ten injections, one six injections, the other two received four injections at intervals of about four days. We were never able to develop an antilytic serum of the potency described by Bordet and Ciuca.

In the following experiment rabbit 1052 was bled after three days after the eighth injection, and rabbit 1098 was bled four days after the sixth injection. The experiment was carried out as follows: Equal mixtures of the immune serum and the Olsen bacteriophage were made, and added immediately and after thirty minutes' incubation and after eighteen hours' incubation. The test was set up as follows:

TUBE	IMMEDIATE BROTH	CULTURE 18	SERUM-BACTERIOPHAGE MIXTURES	READINGS EIGHTEEN HOURS	PLATES AFTER EIGHTEEN HOURS
	cc.	cc.			
1	2	0.5	0.2 cc. mixture Olsen + serum 1052, bled May 23	++ clumped	} Lytic colonies
2	2	0.5	0.2 cc. mixture Olsen + serum 1098, bled May 20	++ clumped	
3	2	0.5	0.2 cc. mixture Olsen + serum 1097 (normal)	++	
4	2	0.5	0.1 cc. mixture Olsen	+	
5	2	0.5	0.2 cc. saline	++	Normal growth

(Turbidity equal to control, indicated by ++.)

The same result was obtained after the mixtures of serum and Olsen bacteriophage were incubated for thirty minutes and for eighteen hours.

One other protocol is of interest in which the immune serum was tested against dilutions of the Olsen bacteriophage, and in which immune typhoid serum was used as a control in order to show that agglutination alone did not interfere with lytic action. In this case the immune serum was added to the typhoid broth first and then the dilutions of the Olsen bacteriophage added. Serum was used from Rabbit 1052, which at that date had received nine injections.

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TUBE	BROTH	CUL- TURE 18	SERUM	OLSEN BACTERIOPHAGE	SIX HOUR PLATES
	cc.	cc.			
1	2	0.1	0.2 cc. 1052 bled July 1	0.1 cc. undiluted	Many lytic colonies
2	2	0.1	0.2 cc. 1052 bled July 1	0.1 cc. 1:100	Many lytic colonies
3	2	0.1	0.2 cc. 1052 bled July 1	0.1 cc. 1:1000	Few transparent areas
4	2	0.1	0.2 cc. immune typhoid horse serum	0.1 cc. undiluted	Many lytic colonies
5	2	0.1	0.2 cc. immune typhoid horse serum	0.1 cc. 1:100	Many lytic colonies
6	2	0.1	0.2 cc. immune typhoid horse serum	0.1 cc. 1:1000	Many lytic colonies
7	2	0.1		0.1 cc. 1:1000	Many lytic colonies
8	2	0.1		0.1 cc. 1:1000	Many lytic colonies
9	2	0.1		0.3 cc. salt	Normal growth
10	2	0.1	0.2 cc. immune typhoid horse serum		Normal growth

The bacteriophage immune serum did not prevent lysis even if the bacteriophage was diluted 1:1000, although it was diminished in tube 3. Typhoid immune serum did not inhibit lysis. The other rabbit gave similar results. In some cases the antilytic serum tended to prevent the clearing up of the bacterial emulsion, but on plating lytic colonies were obtained in every instance. We have not, therefore, been able, like Bordet and Ciuca, to produce an antilytic serum of sufficient potency to prevent lytic action

permanently. We must agree with d'Herelle and Bail that the inhibition of lysis with immune sera prepared by the inoculation of large amounts of dissolved culture filtrate into rabbits is not complete. We have not found that normal rabbit sera interfere with lysis.

Protection experiments

It has been reported that guinea-pigs can be protected against an M.L.D. of culture by the injection of the homologous dissolved culture filtrate, and many workers have used typhoid and staphylococci bacteriophages in treating human cases.

We have performed experiments in which we have injected bacteriophage before and after the infection had begun, and have not found that the injection of lytic filtrate had any very striking advantages over the injections of sterile broth. The M.L.D. of the typhoid culture used was large, the washings of one eighteen-hour agar slant of typhoid being necessary to kill a pig of between 200 and 300 grams. Smaller doses were tried, but failed to kill the control pigs regularly. In the final experiment two pigs were used for each step, a light pig and a heavy pig as far as possible in pairs, the heavier pair in each case being used for controls. Eight pigs were used altogether; 4 pigs were injected with the M. L. D. of culture at 10:30 a.m. After four hours 2 of these pigs were injected with 2 cc. of sterile broth each, the other 2 were injected with 2 cc. of the homologous typhoid dissolved culture filtrate (Olsen). In the other pigs the bacteriophage and the sterile broth were inoculated first, and after an interval of four hours the M. L. D. of culture was injected. The cultures for the animals which received the lytic agent and broth first were stored on ice during the interim, to prevent further growth. All the injections were made intraperitoneally.

January 19, 1922. Animals in which the culture was injected first

	C. P.	WEIGHT	M. L. D. TYPHOID CULTURE	TIME	BROTH OR BACTERIOPHAGE	TIME	RESULT
		<i>gms.</i>		<i>a.m.</i>		<i>p.m.</i>	
Controls	364	330	1 slant no. 18	10:30	2 cc. broth	2:30	Dead 1/20/22, 9 a.m.
	363	245	1 slant no. 18	10:30	2 cc. broth	2:30	Dead 1/20/22, 9 a.m.
Bacterio- phage	369	250	1 slant no. 18	10:32	2 cc. Olsen	2:32	Dead 1/20/22, 9 a.m.
	362	180	1 slant no. 18	10:32	2 cc. Olsen	2:32	Dead 1/19/22, 10 p.m.

Animals in which the bacteriophage was injected first

	C. P.	WEIGHT	BROTH OR BACTERIOPHAGE	TIME	M. L. D. TYPHOID CULTURE	TIME	RESULT
		<i>gms.</i>		<i>a.m.</i>		<i>p.m.</i>	
Controls	400	270	2 cc. broth	10:35	1 slant no. 18	3:00	Survived
	323	210	2 cc. broth	10:35	1 slant no. 18	3:00	Found dead 1/20/22 9 a.m.
Bacterio- phage	325	270	2 cc. Olsen	10:37	1 slant no. 18	3:05	Survived
	394	195	2 cc. Olsen	10:37	1 slant no. 18	3:05	Died in 48 hours

At 4:30 p.m., January 19, 1922, all the pigs were punctured and plates made of the exudate with the following result:

No. 364. Good growth; normal typhoid colonies.

No. 363. Good growth; normal typhoid colonies.

No. 369. Many lytic colonies.

No. 362. No growth.

No. 400. Growth obtained; normal.

No. 323. Growth obtained; normal.

No. 325. Many lytic colonies.

No. 394. No growth.

Lysis was, therefore, going on actively in the peritoneum of the pigs 325 and 369, which had received inoculations of bacteriophage. No growth was obtained from the other two bacteriophage pigs, 362 and 394. Every pig that died was autopsied to make sure that it had not died from any other cause, and typhoid bacilli were isolated from the peritoneal cavity of each. Only two pigs out of the eight survived: one that had been injected with 2 cc. bacteriophage and one that had been injected with 2 cc. of sterile broth four hours before the injection of the M.L.D. of typhoid bacilli.

Conclusions from animal experimentation

We have not been able to confirm the opinion of other workers that the protective action of dissolved culture filtrate is very striking. We were unable to show any very definite advantage in the case of the Olsen bacteriophage over the injection of

sterile extract broth. Bordet and Ciuca obtained definite protection in guinea-pigs against injections of *Bact. coli*. Since guinea-pigs are not susceptible to infections either with typhoid or *Bact. coli* and death in both cases is probably of toxic origin, conclusions as to the value of bacteriophage treatment for human beings cannot be drawn from these experiments.

Study of the leucocytic exudates obtained from guinea pigs in protection experiment

The peritoneal exudates from all the pigs that died were collected, twice the volume of sterile broth added, and the tubes left standing at room temperature for forty-eight hours. At the end of this time they were plated with the following results:

No. 364. Small number of lytic colonies.

No. 363. Many lytic colonies.

No. 369. No lytic colonies, pure typhoid.

No. 362. No lytic colonies, pure typhoid.

No. 323. No lytic colonies, pure typhoid.

No. 394. No lytic colonies, pure typhoid.

Thus the exudates from the two control pigs showed lytic colonies, whereas the exudates from the pigs that had been injected with bacteriophage which had previously shown lytic colonies (no. 369), after standing at room temperature showed nothing but resistant types.

The tubes were kept on ice from January 22 to January 24 and then heated at 59° for thirty minutes. and tested for sterility. These exudates were set up against the stock strain no. 18 as follows:

TUBE	AMOUNT OF BROTH	CUL- TURE 18	PERITONEAL EXUDATES	PLATES AFTER SEVEN HOURS
	cc.	cc.		
1	2	0.1	0.5 cc. no. 364	Few transparent areas
2	2	0.1	0.5 cc. no. 363	Transparent areas
3	2	0.1	0.5 cc. no. 369	Little growth, 1 lytic colony
4	2	0.1	0.5 cc. no. 362	Little growth, 2 normal colonies
5	2	0.1	0.5 cc. no. 323	Normal growth
6	2	0.1	0.5 cc. no. 394	No growth obtained
7	2	0.1	Salt solution	Normal growth

As already stated under the section on the production of bacteriophage by the method of Bordet and Ciuca, it was possible to produce a lytic exudate by a single injection of typhoid bacilli intraperitoneally into guinea-pigs.

VI. IS THE LYTIC PHENOMENON A FACTOR IN RECOVERY FROM INFECTION?

Occurrence of bacteriophage in carrier stools

It seemed of interest in view of the claims made for the beneficial results obtained by d'Herelle in the treatment of dysentery cases with bacteriophage, and by others in the treatment of boils with staphylococcus lytic agent, to determine the incidence of bacteriophage in carrier stools.

Through the courtesy of Dr. Krumwiede of the Research Laboratory of the Health Department, we were able to obtain carrier stools at frequent intervals, and also in one instance a specimen of blood from one of the carriers.

We obtained carrier stools from two typhoid carriers of long standing, Mary Mallon and Mary Newton, residing at the Riverside Hospital, at weekly intervals for a period of six weeks. The fecal specimens were plated on Endo's medium and the homologous strain of typhoid isolated. The proportion of typhoid colonies in the stool of Mallon was consistently less than with Newton. The plates were always carefully examined with the microscope but in no instance were any lytic or abnormal colonies observed.

The fecal suspensions were treated in two ways, the first time the specimen was received: in one case the suspension was thoroughly shaken and filtered immediately, in the other it was shaken and then incubated for four hours and filtered. The filtrates thus obtained from Newton and Mallon were tested against the homologous strain of typhoid and also against the stock strain of typhoid no. 18, since it was thought that the typhoid strain in these carrier stools might be resistant.

February 7, 1922

TUBE	AMOUNT OF BROTH	TYPHOID CULTURE	STOOL FILTRATE	TWO HOUR PLATES
	cc.			
1	2	Mallon	1 cc. Mallon shaken filtrate, February 1, 1922	Many lytic colonies
2	2	No. 18	1 cc. Mallon shaken filtrate, February 1, 1922	Normal growth
3	2	Mallon	1 cc. Mallon shaken filtrate, incubated 4 hours, February 1	Many lytic colonies
4	2	No. 18	1 cc. Mallon shaken filtrate, incubated 4 hours, February 1	Normal growth
5	2	Newton	1 cc. Newton shaken filtrate, February 1	} Normal growth
6	2	No. 18	1 cc. Newton shaken filtrate, February 1	
7	2	Newton	1 cc. Newton shaken filtrate, incubated 4 hours, February 1	
8	2	No. 18	1 cc. Newton shaken filtrate, incubated 4 hours, February 1	
9	2	Mallon	0.2 cc. Olsen bacteriophage	Many lytic colonies
10	2	Newton	0.2 cc. Olsen bacteriophage	Suspicious colonies but not definitely lytic
11	2	No. 18	0.2 cc. Olsen bacteriophage	No growth
12	2	Mallon	Salt solution	} Normal growth
13	2	Newton		
14	2	No. 18		

The stool filtrate of Mallon was definitely lytic for the homologous strain of typhoid, but not for the stock strain no. 18. The Mallon strain of typhoid was not resistant to the Olsen bacteriophage. The filtrate filtered immediately after shaking was as good as the one filtered after four hours' incubation. The filtrate from Newton had no action on the homologous typhoid strain nor on the stock strain no. 18.

The specificity of the Mallon typhoid strain was tried out as shown in the table on page 95.

The tubes were incubated for one hour and then held at room temperature overnight, and plated. The filtrate was again active against the homologous strain of typhoid and against Shiga, but against none of the other cultures tried.

Specificity of the Mallon filtrate, February 1, 1922

TUBE	AMOUNT OF BROTH	CULTURE	FILTRATE	PLATED AFTER EIGHTEEN HOURS AT ROOM TEMPERATURE
	cc.			
1	2	0.1 cc. Mallon typhoid	0.5	Few lytic colonies
2	2	0.1 cc. no. 18 typhoid	0.5	Normal growth
3	2	0.1 cc. Newton typhoid	0.5	Normal growth
4	2	0.1 cc. Rawlings stock	0.5	Normal growth
5	2	0.1 cc. Shiga	0.5	Reduced growth, "appearances"
6	2	0.1 cc. Mt. Desert	0.5	No lytic colonies
7	2	0.1 cc. Typhi Murium	0.5	No lytic colonies

Tube 1 in the last experiment was heated at 56° for thirty minutes to kill the resistant forms, and then the supernatant fluid tried against four typhoid strains to see if the potency of the Mallon bacteriophage was increased in the second generation.

February 11, 1922

TUBE	AMOUNT OF BROTH	TYPHOID CULTURE	SUPERNATANT FLUID	TWO HOUR PLATES
	cc.			
1	2	Mallon	0.5 cc. tube 1, February 9	Lytic colonies
2	2	No. 18	0.5 cc. tube 1, February 9	Normal growth
3	2	Newton	0.5 cc. tube 1, February 9	Transparent areas
4	2	Rawlings stock	0.5 cc. tube 1, February 9	Normal growth

In the second generation the Mallon bacteriophage had extended its activity and was lytic for the Newton strain of typhoid, as well as for the homologous strain.

Bacteriophage in a carrier stool active against a homologous Bact. coli culture

Repeated tests showed that the filtrates from the stool of the carrier Newton had no action on the homologous or other typhoid strains. It seemed of interest, therefore, to determine whether or not the Newton stool might possibly contain a bacteriophage active against some other organism present in the fecal flora. We consequently isolated three different types of lactose-fermenting organisms occurring on Endo plates of the Newton stool, to

Russell's medium and tried the Newton filtrate against them with the following result:

February 12, 1922

TUBE	AMOUNT OF BROTH	CULTURE	STOOL FILTRATE AND CONTROLS	TWO HOUR PLATES	EIGHTEEN HOUR PLATES
1	2 cc.	Newton ty-phoid	1 cc. Newton filtrate, February 10	Normal growth	Normal growth
2	2	Newton ty-phoid	1 cc. salt	Normal growth	Normal growth
3	2	Newton coli (1)	1 cc. Newton filtrate, February 10	Normal growth	Normal growth
4	2	Newton coli (1)	1 cc. salt	Normal growth	Normal growth
5	2	Newton coli (2)	1 cc. Newton filtrate, February 10	Reduced growth ("appearances")	No growth
6	2	Newton coli (2)	1 cc. salt	Normal growth	Normal growth
7	2	Newton coli (3)	1 cc. Newton filtrate, February 10	Normal growth	Normal growth
8	2	Newton coli (3)	1 cc. salt	Normal growth	.

By setting up a second generation from tube 5 definite lytic colonies were obtained with this particular strain. A bacteriophage active against *Bact. coli* (2) has been obtained from the stool of Newton 6 successive times from specimens obtained at 1 week intervals. The growth of this susceptible *Bact. coli* is very characteristic; it forms a large, coarse colony, and in broth has a tendency to sediment out, leaving the supernatant fluid clear. The Newton filtrates have not proved active against the other types of *Bact. coli* present in the Newton stool.

Each specimen of Newton received was plated and contained in every instance a large number of typhoid colonies. The colonies of

Bact. coli (2) were much less numerous, and in two out of the 6 specimens could not be found on the plates.

We have not studied the occurrence of bacteriophage consistently in a sufficient number of carriers to state in what proportion of carriers bacteriophages active against the homologous typhoid strain occur.

Conclusions on work with carrier stools

We have been able to demonstrate a bacteriophage active against the homologous strain of typhoid in the stool filtrates of a carrier of over ten year's standing and also in one that has been positive for nine months. In another typhoid carrier of long standing, we have shown a bacteriophage active against a certain strain of *Bact. coli* occurring in the fecal flora. Specimens from both these carriers consistently contained these particular bacteriophages over a period of six weeks. The proportion of the susceptible organisms in the stool containing bacteriophage seemed to be reduced, and in two instances the susceptible organism could not be found. The presence of bacteriophage in carrier stools may possibly explain the often observed fact, that carrier stools are sometimes negative.

A specimen of blood obtained from one of the carriers (Newton) was examined for the presence of bacteriophage in the circulation, but none was found.

It is worth reporting, furthermore, that the Ida Olsen from whom the Olsen bacteriophage used throughout this paper was isolated in November 1920 when she was in the convalescent stage after typhoid, has also developed into a chronic carrier. Her stools have been positive for typhoid for a period of one and one-half years. A specimen of feces has recently been obtained from her, but we have not so far been able to obtain a bacteriophage from it.

Discussion as to the nature of bacteriophage

Evidence as to the nature of bacteriophage is still inconclusive. D'Herelle and his co-workers are convinced that all lytic phenomena are due to an ultramicroscopic virus, normally

parasitic on the bacteria of the intestinal tract, but capable by adaptation of attacking a large number of organisms.

Kabeshima first brought forward the point of view that the resistance of the lytic agent was such as to rule out living protoplasm, and that the whole manner of action of the bacteriophage suggested enzyme activity. It is obvious that the lytic agent cannot be classified as an enzyme as commonly defined, since it characteristically acts on living rather than dead cells. Furthermore, there is no analogy in the literature of ferments for an enzyme which is quantitatively increased after acting on a substrate.

Bordet and Ciuca's observation that lytic activity could be demonstrated in the leucocytic exudate obtained in the peritoneal cavity of a guinea-pig by the injection of a normal bacterial culture, seemed to point away from the parasitic theory of bacteriophage, indicating that the source of the lytic agent was probably in the bacteria themselves. In the same way, our own experiments in which we produced lysis of typhoid bacilli, transmittable in series by the action of extracts of normal tissue and of normal serum seems to diminish the likelihood of an external parasite, and suggests that the bacteria themselves are the source of the lytic agent. But by far the most striking evidence that the bacterial cell itself secretes bacteriophage under certain circumstances, or liberates it when it disintegrates in a certain way, is the fact that the lytic agent can be demonstrated in old broth cultures in the case of typhoid and dysentery bacilli, and in alkaline washings of eighteen hour agar cultures, in the case of the staphylococcus.

The high resistance to heat and various chemical reagents also renders d'Herelle's theory less likely, but is in itself not sufficient evidence that the lytic agent is not a living thing, since as d'Herelle has pointed out, there are certain forms of life, such as the spores of *B. subtilis*, and an ultramicroscopic virus occurring on tobacco, that are equally resistant.

Again, as an important argument against the filtrable virus conception, we may cite our own experiments as well as those of others in which the lytic principle was developed in old broth

cultures of previously resistant strains, and has been obtained in salt solution washings of young agar cultures derived from normal colonies. These facts cannot be reconciled with the filtrable virus theory without assuming that apparently resistant bacteria may remain carriers for generations of the infecting parasite. From this point of view, since all the cultures used in these experiments were originally derived from the animal body where they may have been exposed to bacteriophage action, it is possible that what we assume to be a normal colony, is only one which is infected to a less degree with the lytic agent. Under certain circumstances the bacteriophage may become dissociated from the bacteria, and then be demonstrable. Therefore, until the strains of bacteria used to show that bacteriophage develops spontaneously in the process of bacterial growth can be definitely shown to be free of a contaminating parasite by the use of the Barber single cell technique, it will not be possible to disprove conclusively the ultramicroscopic virus theory of bacteriophage.

In the present state of our knowledge, however, taking all facts into consideration it seems to us more reasonable to assume the simpler hypothesis that the bacteriophage represents a secretion of the bacteria, produced under certain circumstances, and of the nature of an autolysin. This autolysin, usually liberated in old bacterial cultures, as a consequence of cell disintegration acts as a catalyst which destroys the delicately adjusted equilibrium occurring in actively growing cells between constructive forces and destructive forces, in favor of the latter. Solution of the bacterial cell consequently results and occurs in such a way that more of the autolysin is liberated.

What the significance of the bacteriophage may be from a therapeutic point of view is difficult to estimate at the present time. The fact that a bacteriophage isolated from boils as shown by Callow, is usually not active against the homologous strain of staphylococcus, is not very encouraging. Also the isolation of bacteriophages active against the homologous strain of typhoid from chronic carriers and the development into a carrier of a convalescent case in which there was a typhoid

bacteriophage present, are not easy to correlate with the supposed therapeutic value of the bacteriophage. The data presented are too limited to warrant any conclusions. Progress will probably be made by determining whether bacteriophage phenomena occur in other pathological conditions such as pneumonia and meningitis.

SUMMARY

It has been shown that bacteriophage phenomena with members of the typhoid group can be initiated by means of a variety of agents: normal tissue extracts from guinea-pigs, and normal rabbit sera, and that the lytic agent is, therefore, not necessarily due to any pathological condition. Bacteriophage action has also been obtained with old typhoid broth cultures in the same way as reported by Bail with dysentery. The bacteriophages produced in this way have usually not been active against the homologous strain, but have been active against other strains of the typhoid-dysentery group.

It is suggested that bacteriophage phenomena may possibly have played a part in the observations made by previous workers on the bactericidal titer of normal rabbit serum for typhoid.

High resistance reported by other observers of bacteriophage to acetone and to alcohol precipitation, and also to chloroform and glycerol have been confirmed.

Attempts to prepare antilytic sera of sufficient potency to prevent lytic action completely, were unsuccessful.

Intraperitoneal injections of bacteriophage into guinea pigs have not afforded any very definite protective action against one M.L.D. of typhoid bacilli.

A bacteriophage principle active against the homologous strain of typhoid in two typhoid carriers has been demonstrated. In another typhoid carrier it has been shown that there was no bacteriophage present active against typhoid, but that the filtrates consistently contained one active against a certain strain of *Bact. coli*. In one other case, where a bacteriophage active against the homologous strain of typhoid was isolated during the convalescent stage of the disease, the patient has developed into a chronic carrier.

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THE INFLUENCE OF H ION CONCENTRATION UPON STRUCTURE

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Most observers of Pfeiffer's bacillus make reference to its structural variability. Coccoid or filamentous forms have frequently been found, along with, or replacing, the typical small rods.

Ritchie (1910) isolated an atypical form from spinal fluid and Wollstein (1915) concluded that the species could be divided into long forms and short forms while Bell (1920) more recently separated three forms which he finds after long cultivation to remain true to type. Certain of Bell's types however suddenly changed to long filaments and as suddenly changed back to the typical form; no explanation of the phenomenon is offered. Lacy (1918) and Maitland and Cameron (1921) have reported the isolation of rod and filamentous forms from the same case.

More distinctly germane to the present discussion are the observations of Valerio (1919) who found ovoid, spherical, clubbed and filamentous forms, especially in old cultures, and those of Wade and Manalang (1920) who found distinctly fungoid forms in cultures of *H. influenzae*. The latter workers report that "under certain conditions" very long filaments with various irregular swellings which they regard as reproductive bodies are developed. These forms appeared in a beef infusion-peptone-salt-boullion containing laked sheeps blood and (what is particularly interesting in view of the following discussion) adjusted "to about 1 per cent acid" and sterilized by filtration or by heat at a low temperature.

During the last four years a number of strains of *H. influenzae* have been under cultivation in this laboratory. These have been isolated for the most part from epidemic cases, some from various pathological conditions when no epidemic influenza was known to exist in the community; and several strains have been sent us from other laboratories.¹

1. These cultures were maintained on a standard medium carefully made so that successive lots were as nearly identical as possible. This was an ordinary beef extract peptone agar made up in every case in the same manner, adjusted with NaOH to pH value of 7.4 and a phosphate mixture of this acidity was added to make the finished medium 0.05M phosphate. The medium was autoclaved and cooled to 80°C., enough whole rabbits blood added to make the finished medium 1 per cent blood, maintained for ten minutes at 80°C. and tubed or plated.

On this medium about half of the strains examined exhibited the conventional rod forms 0.2 to 0.3 by 0.5 to 1.0 μ with slightly rounded ends and parallel sides, and a small number of oval or coccoid forms 0.3 to 0.5 μ in diameter (plate 1, 1). Other strains on this standard medium consisted of these uniform short rods along with a few filamentous types 0.2 to 0.5 by 5 to 10 μ , generally somewhat curved and frequently tapering at the ends (plate 1, 2). A small number of strains show the short rods and the filamentous types in about equal numbers. A still smaller number of strains consist predominantly of the filamentous types with only a small number of small rod forms (plate 1, 3). All of these forms usually stain uniformly although with watery fuchsin a few of the small rods show bipolar granules and frequently the filaments show granules at one end or throughout their length. (See table 1 for a summary of the occurrence of these types in 16 strains.)

Agglutination reactions failed to show any group agglutinins corresponding with the morphological types. Anti-serum was prepared for 11 of the strains, representing the four structural

¹ We are indebted to Prof. E. O. Jordan, Chicago; Dr. W. H. Park, New York; Dr. T. M. Rivers, Baltimore, and Professor H. B. Maitland, Toronto, for one or more cultures.

types and cross agglutinations carried out. These results indicated strains 7 and 10 to possess some relationship; strains 2, 3, 4, 5, and 9 to be somewhat related to each other; and strains 6, 8, 11 and Neg. to be unrelated both to each other and to the other strains. Reference to table 1 will indicate that these strains related by agglutination results belong to different morphological groups.

Where a pure strain shows more than one morphological type the several forms appear in a single colony. Smears made from a number of discrete colonies from various parts of a culture surface are always very similar or identical; any one colony usually shows all the structural types present in the culture and about the same percentage distribution in the colony as in the entire culture.

A conspicuous feature of all these strains is the constancy of the morphological types as long as they are maintained upon this standard medium. Many of these strains have been carried through a large number of cultural generations with very little change from their original form. This is conspicuously true of those strains showing originally the characteristic short rods; these produced a few filamentous forms from time to time but none of them show any tendency to change to a predominantly coccoid or filamentous type. This, however, is only true when the medium remains constant.

2. Although exhibiting a considerable degree of structural stability when grown on a constant medium most of these strains are remarkably pleomorphic on certain other media. Various modifications of the chemical and physical state of the medium resulted in morphological changes, but the most conspicuous and constant results were produced by varying the hydrogen ion concentration of the substratum.

Media prepared like the standard medium, described in the previous section, were buffered with phosphates to a range of H ion concentration from the most acid to the most alkaline which will support the growth of this organism. The same amount of fresh blood (1 per cent) was added to each lot, and the medium was heated to 80°C. for ten minutes and tubed or plated.

A number of strains have from time to time been grown on these media of graded acidities and 16 strains were compared in some detail. These strains were subcultured several times from single discrete colonies, while in 2 cases single organisms, isolated by the Barber method, were made the parents of subsequent generations.

All the strains grown upon media buffered to pH values of about 6.8 to 7.8 present the same structural types as described in the previous section (see table 1), i.e., the acidity of the media may be varied through the above mentioned range without effecting conspicuous alterations in the morphology. It is not possible, however, to define accurately the acidity limits of the media for what may be styled normal morphology. Transition from the normal to the abnormal form is a gradual change and moreover the degree of acidity or alkalinity necessary to effect observable modification varies slightly with different strains. The stated acidity limits, however, approximately define the limits of normal structure in the strains studied.

This range of media, pH 6.8 to 7.8 supporting normal morphology at the same time permits very nearly maximum growth of the species. The amount of growth is slightly greater at a point about midway between these limits than at the extremes but the difference is slight. Avery (1918) made his media for maximum growth pH 7.3 to 7.5 and Bell (1920) found pH 7.3 to 7.4 the most desirable reaction.

In acid media, from pH 6.5 to the maximum acidity for growth, all the strains exhibited some structural variation from the normal form and in most strains the change was extreme. The normal forms of the neutral range of media were replaced in part, in some cases entirely, by long filaments, by various coccus-like or yeast-like forms or by a variety of swollen and irregular shaped forms. (See table 1 for a summary of the occurrence of types.)

The filamentous forms range in length from 5 to 50 microns and occasionally to 100 microns by 0.2 to 1 micron; usually they lie in an undulating wave-like position, some are sharply bent, the ends generally are pointed, occasionally square or rounded.

In a few instances branching has been observed. In most strains a considerable number of the filamentous forms show globoid or irregularly oval swellings at the end or along the course of the filament. The filaments, especially the larger ones, stain rather more deeply with watery fuchsin than the normal rods, and frequently present a granular appearance toward one end or throughout the entire length. The large swellings in the filaments always stain irregularly; usually they are vacuolate and frequently show a distinct tendency to retain the Gram's stain (see plate 1, 11 12, 13).

The strains which show the swollen filaments on the acid media also show a considerable number of independent bodies similar in structure and size to the swollen portion of the filaments. Their usual oval form gives them a somewhat yeast-like appearance though they are frequently quite irregular and occasionally show a tendency to branch (see plate 1, 9, 10, 11). Like the swellings in the filaments these bodies usually stain more deeply than the normal rods and usually tend to retain Gram's stain. They always, however, show a considerable irregularity in staining due to vacuoles and the granules. At the same time a few of these yeast-like bodies remain unstained or only very faintly colored.

When these strains are grown upon alkaline media, from pH 8.0 to the maximum alkalinity for growth, equally striking morphological types appear and a larger percentage of the normal rods are, in most strains, replaced by pleomorphic types. The alkaline types closely resemble the acid types of the same strain though the two are generally distinguishable. The filamentous forms on the alkaline media are in some strains long and slender (see plate 1, 7) but usually they are thicker than the corresponding acid filaments, much more curved or wavy and usually more irregular in staining (see plate 1, 4, 5, 6). Swollen filaments and irregular swollen yeast-like forms occur in a few strains (see plate 1, 8) but less frequently than on the acid media.

A striking feature of all these involution forms, both upon acid and alkaline media, is that they are at their maximum in young cultures. It was generally observed that involutions appear after a few hours incubation and are at a maximum in twenty-

four hour old cultures; older cultures have seldom been found to show any greater degree of variability.

It is apparent that these results must depend to a considerable extent upon the stability of the reaction of the media. Rivers (1920) has shown that some strains of *H. influenzae* slightly increase the acidity, other strains slightly increase the alkalinity of blood-broth and Stillman and Bourn (1920) have shown that in blood-broth containing glucose a final pH value of 6.4 is reached by cultures of this organism. The media used in these experiments contain no sugars and are heavily buffered with phosphates and with blood. Potentiometer readings with a hydrogen electrode at various stages in the development of the cultures, moreover, show too slight a change in the acidity during the period of observation to effect the results.

3. Attempts were made to increase the percentage of involutions and the degree of variability from the type forms by continued cultivation upon a medium of an acidity favouring variability. Strain 4, for example, was grown for twenty cultural generations of twenty-four hours each, on blood agar media of three acidities pH 6.0, pH 7.4 and pH 8.5. At the end of this series of generations the cultures on pH 7.4 media were typical small rods as at the start; the cultures on the pH 6.0 media consisted of the same involution types and about the same percentage distribution as at the end of the first twenty-four hour generation on this acidity; and likewise the cultures on the pH 8.5 media presented the same appearances as at the end of the first cultural generation on this media. Other strains gave similar results.

Transfers were made from these cultures which had been for twenty generations on the acid and on the alkaline media, to the standard medium. After a short period of incubation in their first cultural generation the organisms could not be distinguished from those long cultured on the standard medium.

It would appear, therefore, that these morphological variations are produced in direct reaction to the environment and that their appearance is neither appreciably influenced by the previous history of the organism nor do the new forms show any heritable tendency.

TABLE 1

Morphological types of H. influenzae when grown on blood-agar of various hydrogen-ion concentrations

STRAIN	SOURCE	BLOOD-AGAR BUFFERED TO		
		pH 5.5 to 6.0	pH 6.8 to 7.8	pH 8.5 to 8.8
2	Lung	Rods and filaments	All rods	Rods and filaments
5	Nasopharynx	Mostly rods, few filaments, very few yeast-like	All rods	All filaments
6	Nasopharynx	Rods and filaments, very few yeast-like forms	All rods	All filaments
9	Spinal fluid	Mostly rods, few filaments	All rods	Mostly filaments
a	Spinal fluid	Mostly filaments, few yeast-like	All rods	All filaments
b	Spinal fluid	Mostly filaments, few yeast-like	All rods	All filaments
c	Spinal fluid	Mostly filaments	All rods	All filaments
I	Sputum	Mostly filaments	Mostly rods, few filaments	Mostly filaments
J	Sputum	Mostly filaments	Mostly rods, few filaments	Mostly filaments
4	Nasopharynx	Mostly filaments, many yeast-like forms, few rods	Mostly rods, few filaments	Mostly filaments
8	Spinal fluid	Rods and filaments	Mostly rods, few filaments	Mostly filaments
11	Nasopharynx	Rods, filaments, few yeast-like forms	Mostly rods, few filaments	Rods and filaments
Neg.	Sputum	Mostly rods	Mostly rods, few filaments	Rods and filaments
3	Lung	Rods and filaments, few yeast-like	Rods and filaments	All filaments
10	Spinal fluid	Mostly irregular filaments and few yeast-like forms	Rods and filaments	Mostly irregular filaments and few yeast-like forms
7	Spinal fluid	Mostly filaments, few yeast-like forms	Mostly filaments	All filaments

SUMMARY

1. It has been shown that *H. influenzae* when grown upon a standardized blood-agar medium is usually a short rod though many strains show some coccoid or short filamentous forms. Continued cultivation on this standard medium has resulted in very little morphological variation from the initial condition.

2. Extreme pleomorphic forms have been shown to occur in young cultures in media made acid or alkaline close to the growth limiting reaction for the species.

3. The pleomorphic forms occur to the maximum degree after a few hours cultivation on the media promoting their formation and as suddenly disappear when they are transferred to media promoting normal morphology.

The bearing of these results and others of a similar nature upon the life history of bacteria as discussed by Löhnis and Smith, Hort and others, and more significantly, upon the physical chemistry of the protoplasm, will form the subject of subsequent papers.

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EXPLANATION OF PLATE

PLATE 1

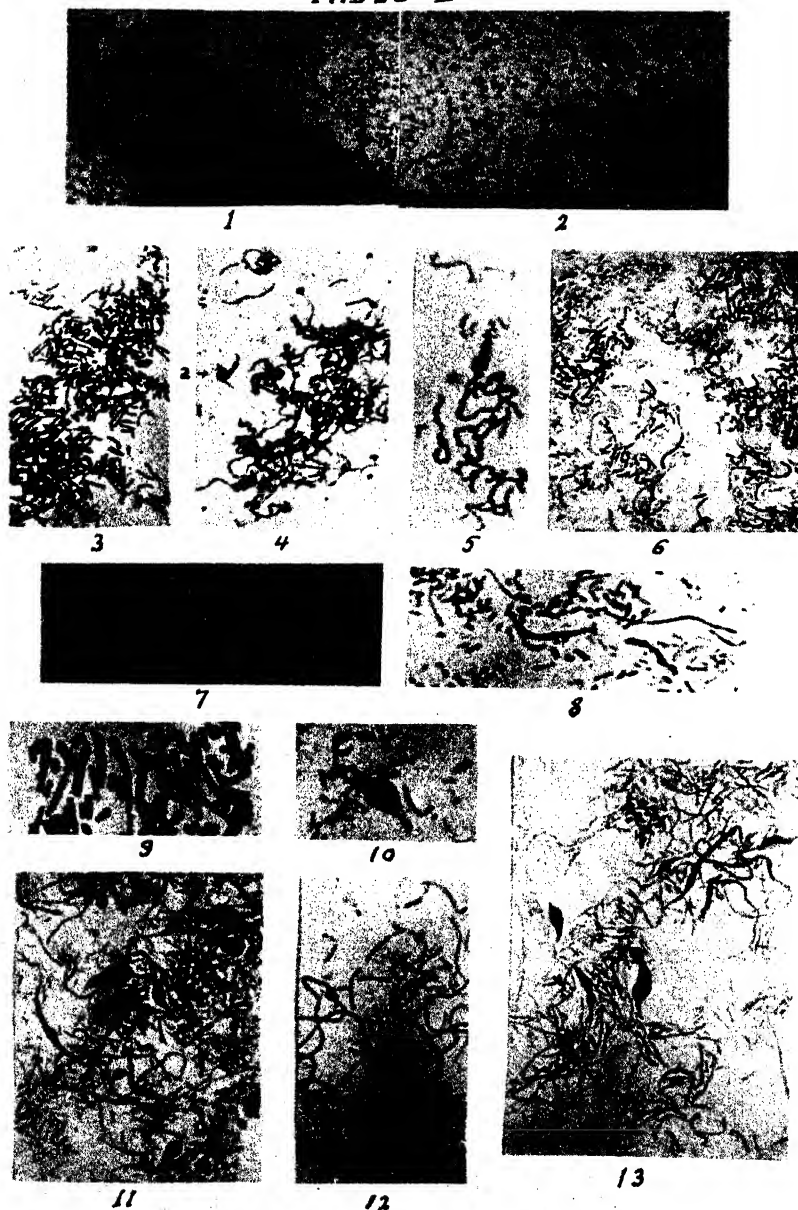
Photomicrographs of smears of *H. influenzae* from cultures on various media, as indicated. The smears in each case were stained by Gram's method counter-stained with watery fuchsin. The magnification in all the figures is the same, 1000 diameters.

1-3. Photomicrographs of smears of different strains of *H. influenzae* grown on blood-agar pH 7.6, the standard blood-agar described in the text. 1, a strain of small rods; 2, a strain of small rods with a few short filaments; 3, a strain of short filaments with a few small rods.

4-8. Photomicrographs of smears of different strains of *H. influenzae* grown on strongly alkaline blood-agar. 4, short tapering filaments with a few swollen types, some showing a tendency to branch, as at *a*; 5, long much twisted filaments. 6, short irregularly stained filaments; 7, very long slender filaments; 8, irregular filaments and rods.

9-13. Photomicrographs of different strains of *H. influenzae* grown upon strongly acid blood-agar. 9, Swollen rods and filaments and a branching yeast-like body; 10, a large yeast-like body showing a bar-like granulation; 11 and 13, rods and long filaments, many much swollen at the ends or in a central position; 12, irregular rods and long filaments.

TABLE I



(Rees and Orr. *H. influenzae*)

VARIATIONS IN HYDROGEN SULPHIDE PRODUCTION BY BACTERIA

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In a paper already published (1921) the writer has called attention to the effect of peptone on indol production. In the present paper there are reported experiments dealing with the effect of peptone on the production of hydrogen sulphide by bacteria.

EXPERIMENTAL WORK

The culture medium employed in nearly all of the experiments was lead acetate agar prepared according to the directions given by Jordan and Victorson (1917), with some modifications, as follows: 3 per cent of peptone was dissolved by boiling in fresh meat infusion; after filtration 1.5 per cent agar was added and dissolved by heating in the autoclave, the reaction was adjusted to pH 7.2 and the medium tubed and sterilized in the autoclave. After sterilization the tubes were cooled to 43°C. and 3 drops of a freshly made 10 per cent solution of lead acetate added to each tube. The tubes were then shaken vigorously and rapidly cooled. The cultures used were grown in peptonized beef broth and the lead acetate tubes were each inoculated with one loopful of broth culture, the loop being introduced between the agar and the wall of the tube and passed down to the bottom. It was found that the best results were obtained when young broth cultures were used for inoculating the agar; twenty-four hour cultures were usually employed, but forty-eight-hour cultures gave results just about as good and were used when more convenient.

The results of a number of such experiments are given below in table 1. Numbers are used to indicate the different varieties of peptone while letters with the numbers indicate different samples of the same varieties of peptone.

Table 1 shows that there are decided differences between the different peptones and between different samples of the same

TABLE 1
Comparative hydrogen sulphide formation with different peptones
Experiment 1

CULTURES	PEPTONES				
	1A	2	3	4A	5
<i>Proteus</i> 4.....	—	+++	—	—	++
<i>Proteus</i> 19.....	—	+++	—	—	++
<i>Bact. suipestifer</i> 360.....	—	—	—	—	—
<i>Bact. suipestifer</i> 416.....	—	+++	—	+++	++
<i>Bact. typhosum</i> A.....	—	+++	—	+++	+++
<i>Bact. typhosum</i> D.....	—	—	—	—	—

Experiment 2

CULTURES	PEPTONES				
	1B	1C	1D	4B	6
<i>Proteus</i> 4.....	+	+	++	+++	+++
<i>Proteus</i> 19.....	+	+	++	+++	+++
<i>Bact. suipestifer</i> 360.....	—	—	—	—	—
<i>Bact. suipestifer</i> 416.....	+	—	++	+++	+++
<i>Bact. typhosum</i> A.....	+	+	++	+++	+++
<i>Bact. typhosum</i> D.....	—	—	—	—	—

— = Negative reaction. + = Weak reaction. ++ = Good reaction. +++ = Strong reaction.

peptone. It also shows that different strains of the same organism may produce different results.

The differing results with different strains of the same organism seemed to warrant more study and as a result there was discovered a curious correlation between hydrogen sulphide production in lead acetate agar and growth in a synthetic medium having the following composition:

Sodium ammonium hydrogen phosphate.....0.2 per cent
 Glucose.....1 per cent
 Brom cresol purple.....5 drops of 0.04 per cent solution per tube

The reaction was not adjusted. The medium was sterilized in ordinary bacteriological tubes at 15 pounds pressure for fifteen to twenty minutes. Growth caused acid production and a change in color from purple to yellow.

TABLE 2

Hydrogen sulphide production by different strains of Bact. typhosum and Bact. suipestifer

ORGANISM	H ₂ S REACTION	SYNTHETIC MEDIUM
<i>Bact. suipestifer</i>		
Number 320.....	Positive	Growth
Number 334.....	Negative	No growth
Number 346.....	Negative	No growth
Number 360.....	Negative	No growth
Number 407.....	Positive	Growth
Number 416.....	Positive	Growth
Number 420.....	Positive	Growth
Number 486.....	Negative	No growth
<i>Bact. typhosum</i>		
A.....	Positive	No growth
HL.....	Positive	No growth
C.....	Positive	No growth
D.....	Negative	Growth
K 110.....	Negative	Growth
K 111.....	Positive	No growth
K 114.....	Positive	No growth

The correlation above mentioned is shown in table 2, which gives the consolidated results of a number of experiments. The peptone used in the lead acetate agar was peptone 2; the results under synthetic medium are those obtained after twenty-four hours' incubation; the results under H₂S reaction represent observations made after one to three days' incubation.

The correlation shown above between the ability of bacteria to produce H₂S and their ability to grow in the synthetic medium appears to be an interesting coincidence rather than evidence of any fundamental connection between the two phases of bacterial

metabolism concerned. As the table shows, the relation between H_2S formation in lead acetate agar and ability to grow in the synthetic medium is exactly opposite in the case of *Bact. typhosum* to what it is in the case of *Bact. suipestifer*. In addition it may be stated that three strains of *Bact. coli* all failed to produce H_2S and all grew in the synthetic medium three strains of *Bact. dysenteriae* all failed to produce H_2S and two failed to grow in the medium while the third grew very slowly, four strains of *Bact. enteritidis* all produced H_2S and all grew in the synthetic medium, eight strains of *Proteus* all produced H_2S and all grew in the synthetic medium, although three were slow in developing, and 15 strains of *Bact. paratyphosum* B all produced H_2S and all grew promptly in the synthetic medium.

It will thus be seen that although ability to produce H_2S and ability to grow in the synthetic medium are not related in a uniform way in the different species of organisms mentioned above, the relation between them is quite constant so far as any one species is concerned.

This is by no means an invariable rule, since exceptions to it are frequently observed. But as far as *Bact. suipestifer* is concerned, out of more than 150 strains recently examined in this division, only 6 failed to conform to the rule that strains which produce H_2S also grow in the synthetic medium while those which do not produce H_2S do not grow in the synthetic medium.

In regard to the relation between hydrogen sulphide production by bacteria and the chemical composition of the various peptones it may be stated that the writer has attempted in connection with the work herein recorded to follow the example of his previous work upon indol production, where everything depended upon the presence or absence of tryptophan, and show that the relative value of peptones for hydrogen sulphide production depended upon the amount of cystin present. It was soon found, however, that the problem was not as simple as it seemed and it was therefore made the subject of a new line of inquiry, the results of which the writer hopes to present before long in another paper.

It can be stated at the present time, however, that experiment has shown that the addition of cystin to lead acetate agar made

with peptone 1 causes the production of H_2S by *Bact. coli* and other organisms which are usually lead negative; while on the other hand the addition of a small amount of sodium thiosulphate makes it possible to obtain results similar in every way to those obtained with peptone 2.

DISCUSSION

The fact that different peptones will yield different amounts of hydrogen sulphide has previously been mentioned by Myers (1920) and by Thompson (1920-21). Each of them used Difco, Fairchild and Witte peptones, which are also included among the 6 used by the present writer. Myers used fluid media while Thompson used a lead acetate agar prepared by a method differing somewhat from that used by the writer. Neither of them has reported any difference between different strains of the same organism so far as H_2S production is concerned. In the case of *Bact. suipestifer* it seems likely that these differences between strains are the cause of conflicting statements in the literature. For example, according to Jordan and Victorson (1917) *Bact. suipestifer* does not produce hydrogen sulphide, while according to Buchanan (1916) and McFarland (1919) it does produce hydrogen sulphide.

The writer has designated the various peptones by numbers and letters instead of giving names, because his object was not to show the superiority or inferiority of any particular varieties of peptones but rather to show what a variable quantity "peptone" really is,—so variable, indeed, that if the names of the peptones were given the information would be more likely to mislead the reader than to help him.

As evidence of this it may be noted that Myers and Thompson ascribe different relative merits to the 3 peptones used by them, while the present writer's work would indicate still different relative values. What else could be expected if we stop to think that, after all, "peptone" in the bacteriological sense of the word is only a name for an indefinite mixture of proteoses, peptones, polypeptides and various other compounds varying enormously

in composition, depending on the materials used and the method of manufacture?

It would seem that until such time as we possess more exact knowledge of the composition of our culture materials, correct results will be possible only if all new culture materials are tested before use and found to be suitable for the particular work to be done.

CONCLUSIONS

1. When used in lead acetate agar different peptones now available in this country yielded extremely variable results so far as hydrogen sulphide production is concerned. In some instances different samples of the same peptone differed from each other.

2. The different strains of *Bact. suispestifer* and *Bact. typhosum* were found to be divided into two groups, those in one group producing hydrogen sulphide while those in the other group failed to produce hydrogen sulphide.

3. On account of variation in the composition of commercial peptones and variation in the hydrogen sulphide producing power of different strains of the same organism, it is advisable before using new culture materials in work with unknown organisms to test these culture materials with known strains of known organisms.

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A COMPARISON OF THE ZIEHL-NEELENSEN AND SCHULTE-TIGGES METHODS OF STAINING TUBERCLE BACILLI

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I have always been impressed with the fact that many patients who exhibit signs of pulmonary tuberculosis clinically and by x-ray plates give a negative test for tubercle bacilli constantly or most of the time.¹ There are, of course, various factors which are beyond control that cause this condition. However, there seemed to be one factor, the staining method used, which might be responsible for some of it. I therefore began to search the literature and found that other workers in this field have been similarly impressed, and have accused the Ziehl-Neelsen method of not always staining the tubercle bacilli; or stating it in another way, that tubercle bacilli are not all acid fast.

In my search I have encountered various modifications of the Ziehl-Neelsen method, and some other methods of staining tubercle bacilli, and the following have been tried:

1. The Hermann method, which is a crystal violet stain.²
2. The Cronberger method, which is a modification of the Ziehl-Neelsen method.³
3. The Schulte-Tigges method,⁴ to be described later.

¹ The work described in this article was done at the United States Public Health Service Hospital no. 32, Washington, D. C., during the months of March, April, May and June, 1921. The method described has been in use at this Hospital ever since.

² A comparison of the Ziehl-Neelsen and Hermann methods of staining tubercle bacilli, *Abst. Bact.*, **3**, no. 1-4.

³ Cronberger method, *Zeitschrift für T.B.*, 1916, **25**, no. 2, p. 109.

⁴ Schulte-Tigges, H. *Deutsche Mediz. Wochens.*, October 28, 1920, **46**, no. 44, p. 1225.

All the above methods have been tested out against the Ziehl-Neelsen method with known positive, negative, and doubtful sputums. In the preliminary tests, it was found that the Schulte-Tigges method gave more encouraging results and it was therefore selected for further study.

The procedure for testing a sputum was as follows: The sputum was well mixed with a glass stirring rod; a portion of it was rubbed between two slides to insure equal distribution of the specimen on both slides; they were dried and then fixed by heat. One of them was stained by the Ziehl-Neelsen method, and the other by the Schulte-Tigges method. The smear should be fairly heavy.

The procedure for staining was as follows:

Ziehl-Neelsen method

1. Flood the slide with carbol-fuchsin (10 cc. of saturated solution of basic fuchsin in 95 per cent alcohol, and 90 cc. of 5 per cent carbolic acid).
2. Heat the slide to steaming and continue the heating for five minutes.
3. Wash the excess stain with tap water, and decolorize in acid alcohol (1 cc. of concentrated HCl in 99 cc. of 70 per cent alcohol).
4. Wash with tap water and counterstain with Methylene blue (saturated aqueous solution of methylene blue).
5. Wash with tap water, dry and examine.

Schulte-Tigges method

1. Flood the slide with carbol-fuchsin (10 cc. of saturated solution of basic fuchsin in 95 per cent alcohol, and 90 cc. 5 per cent carbolic acid).
2. Heat the slide to steaming and continue the heating for *one* minute. Avoid excessive heating.
3. Wash off the excess stain with tap water, and decolorize in sodium sulphite (10 per cent aqueous solution of sodium sulphite). This solution should be renewed at least once a week and if convenient twice a week.
4. Wash *thoroughly* with tap water, and counterstain with picric acid (saturated aqueous solution of picric acid).
5. Wash with tap water, dry, and examine. The tubercle bacilli are red, the background is pinkish yellow.

It should be mentioned here that in the original description of the method the excess picric acid is not washed off; however I found that better results are obtained by washing after counter-staining.

It will be noted that the new method is not more difficult than the Ziehl-Neelsen method.

All the slides when shown to be positive for tubercle bacilli, were rated by the Gaffky method; and a record was made of the average number of organisms per field on an average of ten fields. The Gaffky method of recording the results of positive sputum is as follows:

<i>Gaffky's scale</i>		<i>The values used in this test</i>
I. Only one to four bacilli in whole preparation.....		0.2
II. Only one on an average in many fields.....		0.5
III. Only one on an average in each field.....		1.0
IV. Two to three on an average in each field.....		2.5
V. Four to six on an average in each field.....		5.0
VI. Seven to twelve on an average in each field.....		9.5
VII. Thirteen to twenty-five on an average in each field.....		19.0
VIII. About fifty on an average in each field.....		50.0
IX. About a hundred on an average in each field.....		100.0

During the four months in which this method was being tried out, we examined about 800 sputums, 244 of which were positive with the new stain and only 183 positive with the Ziehl-Neelsen stain, or for every 100 positives by the new method only 75 were positive by the Ziehl-Neelsen. Another important factor is the higher Gaffky reading with the new stain which means more organisms per field. In all this investigation no sputum was even found positive by the Ziehl-Neelsen stain and negative with the new stain, while the reverse was true in 33 per cent of the tests.

Figuring as in table 1, it was found that the total number of organisms of the specimens examined was 657 by the Ziehl-Neelsen method, and 3658 by the Schulte-Tigges method.

Therefore the relation between the two stains is $\frac{3658}{657} = 5.57$, or the new method is more than five times as good.

TABLE 1

Showing the results of the tests with both stains, on specimens from patients 3 and 9

PATIENT'S NUMBER	DATE OF TEST	NUMBER OF ORGANISMS PER FIELD	
		Ziehl-Neelsen	Schulte Tigges
3	May 25, 1921	2.5	5.0
	June 7, 1921	9.5	100.0
	June 13, 1921	0.0*	1.0
	June 23, 1921	5.0	19.0
	June 24, 1921	1.0	9.5
	June 25, 1921	0.5	9.5
	June 27, 1921	0.2	5.0
	July 11, 1921	2.5	19.0
	July 12, 1921	0.0*	5.0
	July 13, 1921	0.5	19.0
Total.....		21.7	192.0

$$\frac{19.20}{2.17} = 8.85$$

9	June 3, 1921	0.2	2.5
	June 13, 1921	0.0*	0.5
	June 23, 1921	0.5	19.0
	June 24, 1921	0.0*	5.0
	June 25, 1921	0.2	1.0
	June 29, 1921	0.5	1.0
	July 1, 1921	0.0*	2.5
	July 7, 1921	0.5	19.0
	July 9, 1921	0.5	9.5
	July 9, 1921	0.5	19.0
	July 11, 1921	0.5	9.0
	July 12, 1921	0.0*	0.5
	July 13, 1921	0.0*	50.0
	July 14, 1921	2.5	50.0
	July 21, 1921	0.0*	0.2
	July 22, 1921	0.5	2.5
	July 25, 1921	0.5	50.0
Total.....		16.9	241.2

$$\frac{241.2}{6.9} = 34.9$$

* Note that this is a negative.

THEORETICAL

It is rather speculative to give any reason for the above results. However, the following points may be discussed here:

1. The acid fastness of the tubercle bacilli

Some people share the opinion that the tubercle bacilli are not all acid fast. Our present work does not seem to corroborate this opinion, and if it does, it is only very mildly. This may be seen from the following table.

TABLE 2

Showing all the negatives (60) with the Ziehl-Neelsen and the corresponding Gaffky's by the Schulte-Tigges method

AVERAGE NUMBER OF ORGANISMS PER FIELD								
Patient number	Z. N.	S. T.	Patient number	Z. N.	S. T.	Patient number	Z. N.	S. T.
1	0.0*	2.5						
3	0.0*	1.0	10	0.0*	0.5	17	0.0*	0.2
	0.0*	5.0		0.0*	50.0		0.0*	0.5
4	0.0*	1.0		0.0*	2.5		0.0*	9.5
				0.0*	19.0		0.0*	9.5
5	0.0*	0.5		0.0*	5.0		0.0*	50.0
			12	0.0*	0.5		0.0*	1.0
	0.0*	0.2		0.0*	1.0		0.0*	5.0
	0.0*	1.0		0.0*	2.5		0.0*	2.5
	0.0*	0.2		0.0*	0.2		0.0*	9.5
7	0.0*	0.2		0.0*	0.2	18	0.0*	9.5
	0.0*	0.2		0.0*	9.5		0.0*	50.0
	0.0*	0.5		0.0*	1.0		0.0*	1.0
	0.0*	0.2		0.0*	0.2	19	0.0*	2.5
			13	0.0*	9.5			
8	0.0*	1.0		0.0*	2.5	20	0.0*	2.5
	0.0*	0.5		0.0*	5.0		0.0*	0.2
	0.0*	19.0		0.0*	0.5		0.0*	0.5
				0.0*	0.2		0.0*	19.0
	0.0*	0.5	16	0.0*	0.2		0.0*	0.2
	0.0*	5.0						
9	0.0*	2.5	17	0.0*	5.0	21	0.0*	0.5
	0.0*	0.5						
	0.0*	50.0		0.0*	19.0		0.0*	9.5
	0.0*	0.2						
Total.....	91.5			133.8				183.1

Grand total = 408.4

408.4

60 (number of negatives)

6.60

In examining the results of table 2, we find that the new method gives, on an average, 6.60 organisms per field, while the Ziehl-Neelsen method gives none per field. However, 6.60 is not far from our figure above (5.57), and it would therefore appear that the negatives are not to be accounted for by lack of acid fastness. It should further be mentioned that 75 per cent of the results fall within the range of 5 and less than 5 organisms per field.

2. The counterstain

In several cases, the Ziehl-Neelsen method was tried by using picric acid as a counterstain, and it was found that in every case it gave somewhat higher results than with the methylene blue counterstain. The counterstain is therefore a responsible factor to some degree.

3. The decolorizer

It seems that the sodium sulphite in decolorizing the slide also clarifies it, and therefore exposes more organisms.

Besides the advantages of this stain in exposing more positives and more organisms per field, it is an advantage to dispose of the use of the alcohol as a decolorizer.

CONCLUSION

1. The Schulte-Tigges method of staining tubercle bacilli is not more difficult than the Ziehl-Neelsen method; it is easier.
2. It gives about 33 per cent more positives.
3. It exposes over five times as many organisms.
4. It does away with the use of alcohol.

PHYSIOLOGICAL YOUTH IN BACTERIA¹

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INTRODUCTION

One of the outstanding features in the development of the biological sciences during recent years has been the conception of the unity of living matter; the recognition of the biological, chemical and physical laws which govern all forms of life.

However, in considering some of the problems of life it is frequently customary to think in quite different terms when viewing these phenomena among unicellular organisms than when the metazoa are considered. For example, the period of youth, as it is known among higher animals, is not ordinarily considered to have a physiological counterpart in those unicellular organisms which reproduce, so far as is known, only by cell division. Probably a fair statement of the current view is that of Mitchell (1912) who says, after describing the process or reproduction by cell division in amoebae: "The two amoebae may be called young animals in the sense that they have just come into existence as new individuals, but nothing in their tissues or characters distinguishes them from their parent. So far as the period of youth has any interest or significance, these animals escape it."

It would seem, *a priori*, that the newly formed bacterial cells should show some physiological characteristics which would differentiate them from more mature cells. It would not, perhaps, be carrying the comparison too far to enquire as to whether the young of an unicellular organism, like the young

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of higher organisms, are more sensitive to the influence of the environment than are the more mature cells of the same species. However, experimental evidence of such a nature, so far as we are aware, is entirely wanting. In fact, the observation of Chick (1908) that young cultures of the paratyphoid organism, under the conditions of her experiments, are more resistant to phenol than old cultures has been interpreted by some as indicating a greater general vigor and resistance in young bacterial cells. We do not think the fact that young cells may not enter so readily into chemical combination with a disinfectant justifies the view that they are more resistant to the physical hazards of life; and it certainly does not seem logical to infer that the young cells are born, so to speak, more perfectly adjusted to the environment than are their elders.

Our knowledge concerning the properties of bacteria has been accumulated mainly through the study of what might be termed mature cultures; that is, cultures twenty-four hours or more of age. As is well known, at this age cultures of most of the best known bacteria have passed their period of most rapid growth and have settled down to a more or less inactive state so far as reproduction is concerned. Whether in this state of reproductive latency there is practically no multiplication of cells, or whether the reproduction and mortality are about balanced, there can be no doubt that the rate of cell division is extremely slow as compared with that of a culture during its period of logarithmic growth, and that the cells of the older culture are relatively of much greater age than are those occurring during the period of maximum rate of multiplication. We have, therefore, attacked this problem by making comparative studies of old cultures, and of young cultures taken during their period of most rapid growth.

EXPERIMENTAL

It is well known that brief exposure to cold above the freezing point of the medium in which the bacteria are suspended causes little or no reduction in the number of bacteria subjected to such treatment. This is verified by the data given in table 1

from tests in which cultures of *Bact. coli* six and eleven days old, grown in 1 per cent pepton at laboratory temperature, were diluted in distilled water at a temperature of 2°C. Plates were made from these dilutions immediately and then again after an one hour exposure at this temperature. It will be seen from the data given in the upper half of table 1 that in neither experiment was there a reduction in the number of bacteria.

For plating in this experiment, as in the other experiments to be reported, the medium consisted of the standard extract pepton agar and the plates were incubated at 33°C. for three days.

TABLE 1

Effects of exposure in distilled water at 2°C. upon mature and young cells of Bact. coli

EXPERIMENT NUMBER	AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
			At beginning	After one hour
1	11 days	Laboratory	607	606
2	6 days	Laboratory	720	710
1	4 hours	37°C.	530	155
2	4½ hours	37°C.	373	247

Turning now to the effect of cold upon young bacterial cells, it will be seen from the data contained in the lower half of table 1, in which actively growing cultures of *Bact. coli* (in 1 per cent pepton at 37°C.) were treated in a similar manner, that there was a distinct mortality among the young bacterial cells when subjected to a sudden cooling.

From the results given in table 1 alone it would not be safe to draw the conclusion that chilling by itself was responsible for the death of the actively growing cells, since we measured, in those cases, the mortality in distilled water dilutions held at low temperature. The experiments were conducted in that manner instead of cooling the original cultures in order to eliminate any possible errors in sampling or in making the necessary dilutions for the counts. It is recognized that a part of the mortality might have been due to the sudden change from a nutrient

medium to distilled water; this, however, does not vitiate in the least the indicated fact that there exist physiological differences between young and mature bacterial cells.

In order to test the effect of cold uncomplicated by such an additional factor, another experiment was conducted in which similar old and young cultures were diluted in sterile 1 per cent pepton instead of sterile distilled water. In this test the dilutions of the cultures in pepton solution were held for three hours at 2°C.; counts were made at the beginning and after the first, second and third hours. As the platings at the different periods were all made from the same broth dilutions, the possibilities of error through sampling and subsequent dilutions were again eliminated; and since the dilutions were made in broth of the same composition as that in which the cultures were grown,

TABLE 2

Effect of exposure at 2°C. upon mature and young cells of Bact. coli

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER OF BROTH DILUTION			
		At beginning	After one hour	After two hours	After three hours
12 days	Laboratory 37°C.	970	930	1,010	970
4 hours		3,420	1,460	1,160	980

there would seem to be in this case no probable detrimental factor concerned other than that of temperature. As is seen from table 2, there was again found to be a marked mortality among the cells of the actively growing culture, while in the old culture there was no measurable decrease.

In an effort to find another treatment of a mild nature which would cause a more marked destruction among young than among older bacterial cells, exposures in dilute solutions of NaCl were tried. Winslow and Falk (1919) have shown that the mortality of *Bact. coli* in water is distinctly increased by the addition of 4.5 per cent NaCl. This, in view of the preceding experiments upon the effect of low temperature, encouraged us to believe that a lower concentration of NaCl might be found which would be distinctly lethal for young cells, while having little effect upon

mature cultures. By using a 2 per cent solution of NaCl in distilled water as a dilution medium it was found that mature cells showed little if any mortality from an one hour exposure at laboratory temperature. In the first four lines of table 3 are given the results of such experiments conducted with cultures which had been incubated twenty-one hours at 37°C., two days at laboratory temperature, and seven days at laboratory temperature; the data obtained with these cultures show little if any effect of such treatment.

Contrary to the results obtained with mature cultures, the data given in the last three lines of table 3 show a very marked

TABLE 3

Effect of exposure in 2 per cent NaCl upon mature and young cells of Bact. coli

EXPERIMENT NUMBER	AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
			At beginning	After one hour
1	21 hours	37°C.	890	770
2	2 days	Laboratory	378	356
3	7 days	Laboratory	1,470	1,410
4	7 days	Laboratory	880	940
1	3½ hours	37°C.	2,530	27
2	3½ hours	37°C.	1,110	72
3	3½ hours	37°C.	2,190	55

mortality among young cells of *Bact. coli* when exposed for one hour at laboratory temperature in a 2 per cent solution of NaCl.

As a further confirmation of the foregoing evidence that newly formed bacterial cells are more delicate than older ones, the effect of a short exposure to heat at a relatively low temperature upon old and young cultures was tried. In table 4 are given results obtained by heating *Bact. coli* in 1 per cent pepton (pH 7.0) to 53°C. for twenty minutes. As is well known, there is a rapid destruction of bacteria at all temperatures above their limit of growth, but an examination of these data shows a much greater mortality among the young than among the mature cells or *Bact. coli*.

The experiments thus far reported were conducted entirely with *Bact. coli* and it is of course recognized that it would not be safe to draw too general conclusions from the work on this organism alone; we have, therefore, made similar tests with a stock culture of *Proteus*. This culture is one of those which was extensively studied by Rogers (1921) and belongs to the type that produces carbon dioxide without hydrogen in its fermentation of glucose. It also ferments maltose and thus should probably be designated as *Proteus vulgaris* in accordance with the nomenclature of Wenner and Rettger (1919). In table 5 are given the results obtained from exposures of young and mature cultures of *Proteus* in 2 per cent NaCl solutions for one hour at laboratory temperature. In this case there was no

TABLE 4

Effect of exposure at 53°C. for twenty minutes upon mature and young cells of Bact. coli

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER		RATIO BEFORE : AFTER
		Before heating	After heating	
7 days	Laboratory 37°C.	1, 470, 000, 000	97, 000, 000	15:1
3½ hours		27, 800, 000	24, 600	1, 130:1

decrease in the number of cells from the older culture whereas the young cells showed a definite mortality. Although the contrast in this case was not so marked as in the experiments reported above with *Bact. coli*, it is probable that by increasing the salt concentration, or lengthening the time of exposure, just as striking results could be obtained with *Proteus vulgaris*.

An experiment made upon the effect of mild heating upon *Proteus vulgaris*, which is reported in table 6, gave data confirming those obtained in the studies with *Bact. coli*. In these tests cultures three and one-half and twenty-six hours old at 37°C. (in 1 per cent pepton, pH 7.0) were heated to 50°C. for ten minutes. An examination of the results of plate counts made before and after this treatment shows again a greater mortality in the young than in the mature culture.

Since there appears to be a difference between old and young bacterial cells with respect to their ability to withstand certain deleterious physical agents, it would seem hopeful to attempt to find some differences in the properties of their protoplasts which could be detected by some convenient test. The isoelectric point suggested itself as a possibility, so young and old cultures were tested for their agglutinability with acid. This worked well since the young cells proved to be very resistant to agglutination while the mature cells were readily flocculated when the

TABLE 5

Effect of exposure in 2 per cent NaCl upon mature and young cells of Proteus vulgaris

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
		At beginning	After one hour
<i>hours</i>	°C.		
26	37	391	396
3½	37	1,120	570

TABLE 6

Effect of exposures at 50°C. for ten minutes upon mature and young cells of Proteus vulgaris

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER		RATIO BEFORE : AFTER
		Before heating	After heating	
<i>hours</i>	°C.			
26	37	440,000,000	183,000,000	3:1
3½	37	10,100,000	116,000	87:1

hydrogen ion concentration of the medium was increased to a pH value of about 3.8.

The results of a typical experiment are given in table 7. In this test the cultures were grown in 1 per cent pepton-0.20 M NaCl broth; the old culture was diluted sufficiently with sterile broth to give it about the same turbidity as the young culture; and the agglutination was effected by the addition of N/14 H₂SO₄ to the desired pH value. Readings were made after one and one-half hours at laboratory temperature.

Similar results have been obtained with cultures grown in broths of other compositions as well as with cells taken from agar slopes and suspended in 1 per cent NaCl solution.

The fact that a difference exists in the acid agglutinability of young and mature bacterial cells is, we think, significant. However, the demonstration of this point is not new since MacGregor (1910), studying serum agglutination, found that meningococci grown for more than forty-eight hours became so easily agglutinable as to flocculate with normal serum; while Gillespie (1914), working on the acid agglutination of pneumococci, found young cultures to be much more resistant to agglutination than were older ones.

We are aware of the fact that *Bact. coli* has been credited by some investigators as being non-agglutinable with acid; con-

TABLE 7
Acid agglutination of Bact. coli

AGE OF CULTURE	AGGLUTINATION AT pH VALUE OF				
	6.0	4.1	3.8	3.4	3.0
<i>hours</i>					
4	—	—	—	—	—
24	—	+	+++	+++	++

cerning this point, no comment is called for in the present case other than to state that the culture employed was of the typical colon type with respect to its gas ratio (Rogers, Clark and Davis, 1914), the methyl red test (Clark and Lubs, 1915), and the Voges-Proskauer reaction (Levine, 1916).

While it was not considered that the points established in this work are irreconcilable with those of Chick (1908) upon the destruction of *Bact. paratyphosum* by phenol, it seemed desirable to touch on that point in our work. Table 8 gives the data obtained in one experiment in which the rate of destruction of four and twenty-four hour cultures of *Bact. coli* in 0.5 per cent phenol was tested. In this experiment the cultures were grown in 1 per cent pepton and the phenol added to the pepton culture. The twenty-four hour culture was diluted about one hundred

times with sterile 1 per cent pepton, before the phenol was added, in order to have approximately the same number of cells present in each case and also to have, so far as possible, the same conditions with respect to reaction and metabolic products.

These results indicate that, in the case of the organism studied, the young cells are more sensitive to phenol than are the mature ones. A repetition of the experiment, using the same technique, gave similar results.

In reviewing the work of Miss Chick it is noted that she did not find the young cultures of the paratyphoid organism more resistant to phenol when tested after three hours incubation on

TABLE 8

Action of 0.5 per cent phenol upon young and mature cells of Bact. coli

TEST NUMBER	AGE OF CULTURE	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
		Before treatment	After three minutes in 0.5 per cent phenol
1	<i>hours</i>		
	4	1, 240, 000	300
	24	4, 650, 000	42, 600
2	4½	2, 400, 000	430
	24	6, 900, 000	10, 800

the *first* transfer, but when the *third* generation of three hour cultures was tested they appeared to be more resistant. Since the amount of inoculation used in transferring the cultures was not given, and since the growth curve was not followed, it is difficult to interpret her results in terms of our own. However, it is not impossible, in view of the fact that transfers from actively growing cultures give no lag (Barber, 1908), that the cultures in Miss Chick's experiments had completed the period of rapid multiplication. As Chesney (1916) has well said, "it would seem necessary to obtain accurate knowledge of the growth curve of a culture before the terms 'old' or 'young' are applied to it."

DISCUSSION

The demonstration of a definite physiological youth in bacteria is in full accord with modern developments in general biology as well as with the established facts of bacteriology. The work of Child (1915) establishes definitely the fact that the condition of physiological youth and the process of rejuvenescence may be entirely divorced from sexual processes. His demonstration that regenerating parts of a planarian are physiologically younger than the original organism and that old planarians can be rejuvenated by starvation show clearly that youth in a physiological sense is not necessarily the result of a sexual union. In keeping with this also is the work of Woodruff and Erdmann (1914) with non-conjugating paramecia. In their studies it was found that when a culture of paramecium was carried through successive generations without conjugation the organisms were periodically observed to undergo an internal reorganization process during which the cellular contents were morphologically disintegrated and then redifferentiated. This process, which was named by Woodruff and Erdmann endomixis, appears in a sense to rejuvenate the organisms since it is followed by a more rapid multiplication rate, as is the case following conjugation.

It is perhaps going too far to regard endomixis as a rejuvenating process, especially in view of the recent results of Woodruff (1921) with a new species, *Paramecium calkinsi*, which did not undergo endomixis when carried for a year without conjugation, notwithstanding the fact that it exhibited characteristic growth cycles. Whether this is true or not, the work of Child appears to leave no doubt as to the fact that physiological youth can be achieved by other means than sexual processes.

It would now seem that the conception of young tissue in a physiologic sense should be extended to the organisms which reproduce by simple cell division, the newly formed cells being younger than the parent cell from which they were derived. Such a view is indeed already held by Child (1915) as is indicated by the statement that, "it is certain that the new individuals which arise by division or budding from other individuals or

from experimentally isolated pieces are to some extent physiologically younger than the parent individual from which they arose." The present work, we believe, adds experimental weight to such a view. That morphological differences exist between bacterial cells from mature cultures and from cultures during the period of rapid growth has been well established by the careful works of Clark and Ruehl (1919) and Henrici (1921); that physiological differences should exist would seem only logical in view of the known morphological facts.

Recognition of the fact that young bacterial cells may differ considerably in physiological characteristics from cells of mature cultures should lead to a number of applications in bacteriology. We have found, for example, that the errors which are encountered in the plate count may be quite different dependent upon whether the sample under examination is from a mature culture or from one during its period of rapid growth.

Since the young of bacteria, like the young of higher forms of life, are more susceptible to the hazards of their environment, it is not improbable that in the struggle for existence among these organisms there occurs a certain mortality among the young cells—an "infant mortality" so to speak. If such exists, another factor must be considered in discussions of the relative merits of direct and cultural methods of bacterial enumeration.

As it is probable that the observed differences between young and old bacterial cells are in some way associated with differences in permeability and water content it would seem likely that they would differ in their abilities to stimulate antibody production; such a difference might find ready application in the production of active immunity. The applicability of these facts in agglutination phenomena is already known to some extent and probably deserves wider appreciation.

To cite another example, in an entirely different field of bacteriology, the pasteurization of milk may be mentioned. As has been shown by Ayers and Johnson (1913), the efficiency of pasteurization can not be judged, as was formerly thought, on the basis of the percentage of bacteria destroyed. It is found that in the case of milks with high bacterial counts (in which

there has necessarily been an extensive growth of bacteria) it is easy to obtain an efficiency above 99 per cent, while with milks of low bacterial count (in which there has been little or no proliferation of cells) no such percentage destruction is obtained. It is of course recognized that this fact is probably due in the main to the differences in the types of bacteria predominating in these classes of milks, but it is possible that the physiological condition of the cells with respect to age also plays some part.

SUMMARY

The results of experiments are reported which indicate that physiological differences exist between young and mature bacterial cells; that the newly formed cells pass through a period of physiological youth.

Tests with *Bacterium coli* have shown that cells taken from a culture of this organism during its period of rapid growth are sensitive to certain mild exposures which cause no mortality among the cells from an older culture. Treatments which have yielded such results are brief exposures to cold (2°C.), and exposures in a dilute solution of NaCl (2 per cent).

Experiments conducted upon the effect of heating to relatively low temperatures showed that young bacterial cells are more rapidly destroyed than are the older ones. Other tests indicated that the same is true of the action of phenol.

That there actually exists a difference in the physico-chemical condition of bacterial cells of different ages is indicated by differences in their agglutinability, as was revealed by acid agglutination tests.

Repetition of some of these experiments in which young and old cells of *Proteus vulgaris* were used instead of *Bacterium coli* gave similar results with this organism.

The biological significance of these results and some of their possible practical applications are discussed.

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STUDIES ON SALT ACTION

VI. THE STIMULATING AND INHIBITIVE EFFECT OF CERTAIN CATIONS UPON BACTERIAL GROWTH¹

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The object of the present study was to conduct a general survey of the effect of various cations upon the growth of bacteria, under somewhat widely varied conditions of concentration. Previous work, except that of Lipman (1909), Brooks (1920), Winslow and Falk (1918, 1918a), Falk (1920), and Holm and Sherman (1921), dealt only with limiting toxicities and ignored possible stimulating effects; and the investigators cited studied only a very few salts. It was felt that it would be of real interest to undertake a more comprehensive survey of the stimulating, as well as the toxic, concentration of the various chemical elements. A series of inorganic compounds was used in which different cations were combined with the same anion. At it is presumed that materials in solution are most likely to be reactive with protoplasm, the chlorides were chosen because they form a large series of soluble compounds and because the anion is a single element.

The organism which was used in these studies was the *Bacterium coli* strain which has been used by Winslow and Falk (1918, 1918a) and by Cohen (1922). From its cultural and morphological characteristics it may be grouped as a *communis* type of *Bacterium coli* (sucrose negative, dulcitol positive).

In order that a wide range of chemical elements might be studied it was necessary to develop a rapid method for the determination of the number of bacteria per cubic centimeter.

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It was decided that the simplest method for the estimation of bacteria was by a comparison of relative turbidities when the organisms were grown in a fluid culture medium, a procedure used with success by Holm and Sherman (1921).

It was necessary to provide a basic fluid medium with some buffer action and one which would be of sufficient complexity to produce a bacterial growth which would give measurable degrees of turbidity. For this purpose a one per cent solution of Bacto-peptone (Digestive Ferments Co.) was finally chosen. Ash determinations gave the inorganic content of the dried peptone as 4 to 5 per cent, so that a 1 per cent solution of the peptone contained about 0.05 gram of inorganic material for 100 cc. of solution. A communication from the Digestive Ferments Company stated that the ash was principally a sodium one, and that calcium oxide, phosphorus anhydride, sulphur and a small amount of chloride were also present.

Other media, of known chemical composition and lower ash content, did not prove useful as culture media. At first, growth studies were made with Dolt's synthetic media, composed of asparagine and di-basic sodium phosphate or asparagine and di-basic ammonium phosphate. These media were not sufficiently favorable to bacterial growth to produce marked changes in turbidity. A peptone mixture prepared by the hydrolysis of dialyzed edestin had a low ash content, but this product also gave insufficient bacterial growth.

In the development of a scheme of turbidity measurements it soon became apparent that accuracy could better be obtained through an average of many readings than by an attempt at mathematical precision through the use of turbidimeters or other optical devices. To estimate the growth, standard suspensions of bacterial cells, killed by heating at 56°C. for one hour, were used. The number of bacteria per cubic centimeter was determined by plating equivalent dilutions of the suspension of bacteria before heating. The standards were sealed with paraffin and kept at ice-box temperature; they did not deteriorate for a period of from six weeks to two months. Five sets of standards were prepared during the period of the study, each set with

approximately the same range of turbidities as determined optically and by the plate method. The relation between turbidity readings and bacterial numbers, recorded in millions of bacteria per cubic centimeter, are based on an average of the plate counts for the five sets of standards. Turbidities which are produced by more than 1200 million bacteria per cubic centimeter are difficult to read accurately and the figure 2700 was used to cover a general range between 2000 and 3500 million bacteria per cubic centimeter. The tables and charts in this article were prepared from figures obtained by averaging the turbidity readings of different tests.

With some of the less toxic salts a variation in the seeding of the tests might have caused a variation in the toxicity point; therefore it was necessary to inoculate with a uniform amount of culture. The stock culture of *Bact. coli* was grown in 1 per cent peptone solution. In inoculating the test fluids 1 cc. of a twenty-four-hour culture was added to 10 cc. of sterile water and with a pipette graduated to 0.01 cc. the culture was transferred in amounts of 0.05 cc. Thus in a single test each tube received the same amount of culture as its salt free control, although tests prepared at different times might have received slightly different amounts due to a variation in growth of the initial culture.

Efforts were made to use chemicals as free from impurities as possible. The sodium, potassium, calcium, barium and mercuric chlorides were purified by re-crystallization from distilled water. The ammonium, magnesium and strontium chlorides were Baker analyzed. The aluminium, cupric, lead, nickel and zinc chlorides were from Merck and Co. They were of 'C. P.' quality but were not re-crystallized. The cadmium, cobalt, ferric and ferrous, manganese and stannic chlorides were from Eimer and Amend, of tested purity with analyses. The cerium, lithium, thallium and titanium chlorides were from the same company, of 'C. P.' quality but not labeled with the analyses.

Salt solutions of known molar concentration were prepared, except in the case of titanium chloride, by adding carefully weighed amounts of salts to the proper amount of distilled water. The titanium chloride was supplied in solution, so that

the stock molar concentration was prepared from it by dilution. In all work on which conclusions are based anhydrous salts were used or the water of crystallization was allowed for in the calculations.

In order to obtain a series containing a constant 1 per cent peptone and varying molar concentrations of the salts, a solution containing 2 per cent peptone was used, from which by dilution with distilled water and stock salt solution the final product of the desired concentration was prepared. Calculated amounts of peptone solution and distilled water were added, by burette and pipette, to a series of bottles. The desired amount of stock salt solution was then added by pipette to one bottle and from this bottle the other solutions were prepared by dilution. The uniform results obtained tend to show that the solutions thus prepared did not vary appreciably.

Salts such as sodium and potassium chlorides and calcium and magnesium chlorides were added to the peptone solution as described, tubed in 5 cc. amounts and sterilized by autoclaving. Other salt solutions which could not be heated because of decomposition were prepared in the following manner: the stock salt solutions were made up and stored until they became sterile; the proper amount was then transferred with a sterile pipette to peptone dilutions which had previously been autoclaved. The solutions were then carefully poured into sterile test tubes calibrated to 5 cc. Contaminations occurred but rarely and were easily recognized by the appearance of a pellicle and by distinctly heavier growth. From all suspicious tubes plates were poured and microscopic examinations for spore-bearing organisms were made.

When added to the peptone solution, ammonium, calcium, lithium, magnesium, potassium or sodium chlorides caused no visible change. Salts which underwent hydrolysis with the formation of an insoluble hydroxide gave a precipitate in solution; and such solutions were shaken thoroughly before taking a sample with the pipette.

Barium chloride and many salts of the heavy metals gave a very troublesome precipitate when added to the peptone solution.

As the solutions could not be sterilized after preparation no attempt was made to filter the solutions and the lower concentrations were prepared by transferring a portion of the first prepared dilution, containing both solution and precipitate. In such cases the actual concentration of salt in solution in the final tube is unknown, except that it was not above the amount added.

Each set of test solutions consisted of four tubes of each dilution, three of which tubes were inoculated; and three inoculated tubes of a 1 per cent peptone solution were used as a control. The fourth tube of salt solution was used for an initial hydrogen ion determination.

The hydrogen ion content of the solutions was determined before inoculation and after growth had continued for ten days. The indicator method of Clark and Lubs was used. This method proved applicable even to salts which produced colored ions, as these salts were so toxic that the dilutions used were very high.

In the comparison of the test cultures with the standard bacterial suspensions direct and reflected light were used. Before comparison the tubes were shaken in order that any sediment might be distributed. Chemical precipitates did not usually interfere with the readings as growth did not ordinarily occur in concentrations where a precipitate was formed. There were some exceptions to this rule, barium chloride being especially troublesome.

To facilitate comparison with the standards a wooden block, painted black, with spaces for twelve test tubes in double rows was used. Rectangular openings, of about 0.5 cm. by 2 cm., extended from side to side of the block and permitted light to pass through the tubes. Behind the standard tube was placed a tube containing 1 per cent peptone solution and tubes of water were placed behind the test cultures to render the optical effect the same.

All tests of the growth of the bacteria in salt solutions were made at an incubation temperature of 37°C.

EXPERIMENTAL WORK

The salts studied were:

Sodium chloride.....NaCl	Nickel chloride.....NiCl ₂
Potassium chloride...KCl	Thallium chloride....TlCl
Lithium chloride.....LiCl	Cupric chloride.....CuCl ₂
Ammonium chloride...NH ₄ Cl	Ferric chloride.....FeCl ₃ . 12 H ₂ O
Strontium chloride...SrCl ₂ . 6 H ₂ O	Ferrous chloride.....FeCl ₂ . 4 H ₂ O
Magnesium chloride...MgCl ₂ . 6 H ₂ O	Zinc chloride.....ZnCl ₂
Calcium chloride.....CaCl ₂	Cobalt chloride.....CoCl ₂ · 6 H ₂ O
Barium chloride.....BaCl ₂	Lead chloride.....PbCl ₂
Manganese chloride...MnCl ₂ . 4 H ₂ O	Aluminium chloride...AlCl ₃
Titanium chloride....TiCl ₃ (15 per cent solution)	Cerium chloride.....CeCl ₃
Stannic chloride.....SnCl ₄ . 5 H ₂ O	Cadmium chloride....CdCl ₂
	Mercuric chloride.....HgCl ₂

The salts divide into two general groups, one group with which no growth occurred in concentrations of 2 to 0.05 molar and a second group, where dilutions of 0.01 to 0.00001 molar were sufficient to prevent growth. As might be expected, those salts which are of common occurrence in the protoplasmic environment form the non-toxic group. They are the salts found in the first column above.

Sodium chloride may be studied as a typical mono-valent salt of group I. When the test cultures were examined at the end of twenty-four hours' incubation, growth was seen in all tubes which had a concentration of less than 1.0 M. The growth formed a fine evenly distributed suspension. There were no clumps in any dilution and when the tubes were shaken little or no sediment rose. A difference in turbidity existed in the different dilutions; those tubes which had a concentration of more than 0.25 molar showed less turbidity. The 0.25 molar concentration showed a relatively greater turbidity. At the end of two and three days' incubation the superiority of the 0.25 molar concentration for growth was even more marked, both higher and lower concentrations showing less turbidity than was observed in this solution. This gives a peak in the curve when the molar concentrations are plotted against the number of bacteria present as measured by turbidity. At the end of ten days this peak is obscured, for the organisms in the concentrations below 0.25

have usually multiplied until their turbidities equal those produced in the 0.25 molar concentration. The growth in the molar, 0.75 molar and sometimes the 0.5 molar concentrations showed a lag in comparison with the other concentrations. Those concentrations which showed a small amount of growth at the end of ten days contained little sediment, while concentrations which produced heavy growth showed more sedimentation.

Potassium chloride presented almost the same picture; and the curve of growth for ammonium chloride was the same. The growth picture was slightly different, here, as the organisms

TABLE 1

Average of readings for three days incubation period. Sodium, potassium, lithium and ammonium chlorides. Million bacteria per cubic centimeter

CONCENTRATION	NaCl	KCl	NH ₄ Cl	LiCl
4.0 M.	0			
3.0	0	0		
2.0	0	0	0	0
1.0	140	180	0	0
0.75	360	250	400	0
0.5	700	480	740	150
0.25	1700	1200	2700	500
0.125	1000	950	1100	600
0.05	1000	950	980	600
0.025	950	950	820	600
0.0125	820	950	700	450
0.005	850	950		
Control	900	800	500	500

showed a tendency to grow in clumps, and so to sink to the butt of the tube and form sediment. Lithium had not such a distinct optimum point but an optimum range between 0.25 and 0.05 M was noted.

If an average of the readings made at different concentrations after three day periods of incubation is taken, growth curves for the four salts are obtained which may be seen in table 1 and chart 1. The molar concentrations are the abscissae, plotted on a logarithmic scale against the turbidity readings as ordinates, plotted to the same scale. The heavy vertical line at the right

of the charts indicates a 1 molar concentration. The curves for sodium, potassium and ammonium show a rapid rise to an optimum at 0.25 molar concentration and a tendency to sink to a lower level of growth which is maintained, at this incubation period, in the remaining low dilutions. The optimum occurs at exactly the same concentration for all these salts.

All of these salts except ammonium chloride form solutions which are nearly neutral (pH 6.6–6.8). They are composed of elements which are strong bases and strong acids. The pH of the 2 M solution of ammonium chloride was 6 and that of all the less

TABLE 2

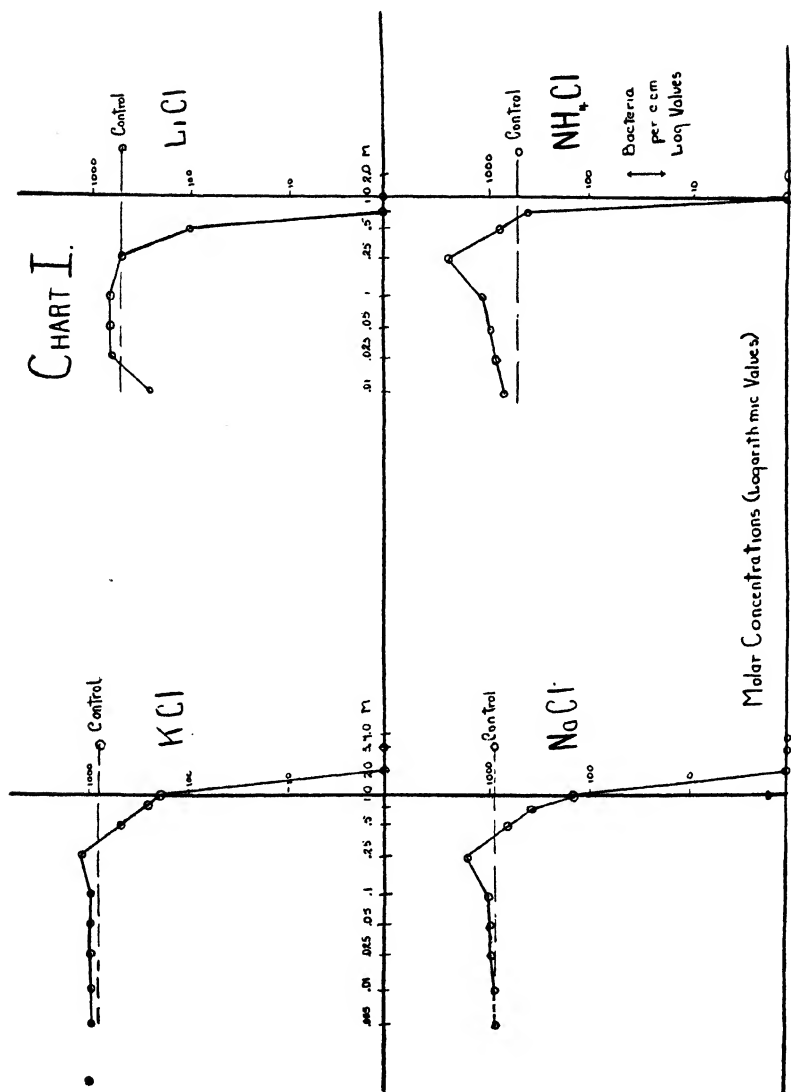
Average of readings for three days incubation period. Strontium, calcium, magnesium, barium and manganese chlorides. Million bacteria per cubic centimeter

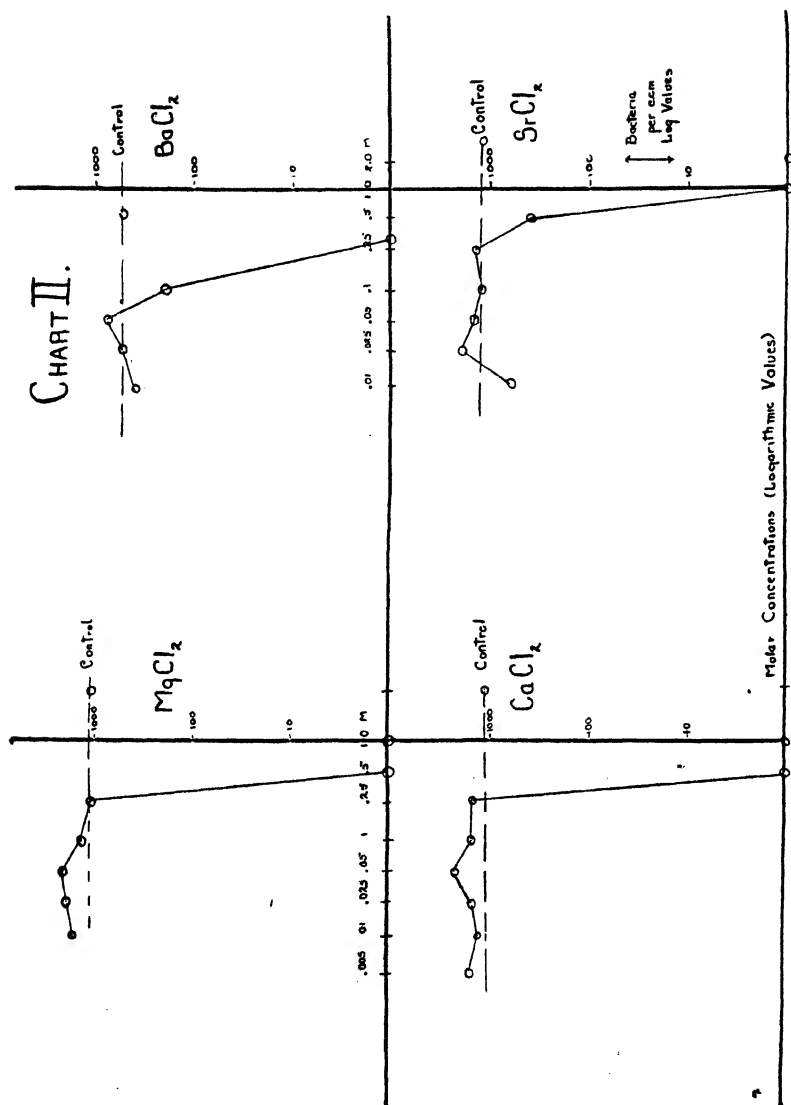
CONCENTRATION	SrCl ₂	CaCl ₂	MgCl ₂	BaCl ₂	MnCl ₂
1.0 M.	0	0	0	0	0
0.75		0	0		0
0.5	400	0	0		0
0.25	1450	1460	1130	0	0
0.1	1200	1530	2030	200	0
0.05	1570	2240	2200	800	0
0.025	1950	1500	1860	550	400
0.0125	700	1330	1700	400	400
0.005		1700			
Control	1250	1100	1160	550	700

concentrated solutions was 6.4. The peptone solution gave a pH of 6.8–7.0.

After ten days' incubation the concentrations which inhibited growth showed little or no change in hydrogen ion concentration. Tubes in which growth occurred, on the other hand, became very alkaline due to substances produced during metabolism. Thus the pH of the NaCl solution after growth was complete averaged 8.2; that of the KCl, 8.5; that of the NH₄Cl, 6.7; that of the LiCl, 7.8.

The bi-valent salts of group I were more toxic than the mono-valent salts. Strontium chloride was the only one which ever

CHART I. GROWTH OF *Bacterium coli* AS INFLUENCED BY SOLUTIONS OF FOUR MONOVALENT SALTS

CHART 2. GROWTH OF *Bacterium coli* AS INFLUENCED BY SOLUTIONS OF FOUR BIVALENT SALTS

showed growth in 0.5 molar concentration and in two tests out of four there was no growth in this concentration. Barium, calcium, magnesium and manganese were all toxic in 0.5 molar concentration. With the exception of manganese an optimum concentration point was found where growth was better than in the peptone control. This optimum occurred in concentrations ranging from about 0.05 M to 0.025 M. The maximum point became obscured after an incubation period of three days.

There was a tendency for the bacteria to grow in clumps and at the end of ten days' growth there was much sediment with clearing of the solution. This was in marked contrast to the peptone control tubes and the tubes containing sodium and potassium where the organisms formed a homogeneous suspension.

Barium chloride formed a precipitate with the peptone solution in the dilutions used. The turbidity readings were, in this case, supplemented by observations on agar slants streaked with a loopful of the test solution.

The hydrogen ion concentrations were, in general, not different from concentrations found in solutions of the uni-valent salts of group I; although manganese showed a greater initial acidity in molar solution than any of the salts thus far reported. The pH was 5.8 rising to 6.8 at the 0.05 molar concentration, while the other solutions gave an initial pH 6.6–7.0. After the completion of incubation (ten days) those tubes in which growth had occurred showed the following averages: SrCl_2 , pH = 8.2; CaCl_2 , pH = 7.9; MgCl_2 , pH = 7.4; MnCl_2 , pH = 7.5.

The second group of salts studied comprises the salts of the heavy metals, which were found to be more toxic.

Group II

	AlCl_3	HgCl_2
	CdCl_2	NiCl_2
	CeCl_3	PbCl_2
	CoCl_2	SnCl_4
	CuCl_2	TiCl_3
•	FeCl_3	TiCl
	FeCl_2	ZnCl_2

Several conditions made this group difficult to study. The first was the difficulty of preparation, due to the fact that the

solutions could not be autoclaved. The method by which this difficulty was overcome has been discussed above; it provided a chance for contaminations, but such contaminations were easily recognized in the rare instances in which they occurred.

An examination of the hydrogen ion concentration data (table 3) shows very high acidity in even low concentrations of these salts, due to hydrolysis. The salts of group II are formed by

TABLE 3

*Hydrogen ion concentration before incubation. Salts of the heavy metals.
pH readings*

CONCENTRATION	AlCl ₃	CdCl ₂	CeCl ₃	CoCl ₂	CuCl ₂	FeCl ₃	FeCl ₂
0.01 M			6.0		4-5		2.0
0.005		6.4		3-4	4-5	4-5	
0.001	4-5	6.8	6.5	6.6	6.0	-6.0	4-5
0.0005	4-5	6.8	6.4	6.8	6.4	6.2	6.0
0.0001	6.6	6.8	6.6	6.8	7.0	6.4	6.8
0.00005		6.8	6.6	7.0		6.0	6.8
0.00001		6.8	6.6	7.2		6.8	6.8
0.000005		7.0	6.6				7.0

CONCENTRATION	HgCl ₂	NiCl ₂	PbCl ₂	SnCl ₄	TiCl ₃	TiCl	ZnCl ₂
0.01 M	5.0	4-5		4.4	4.4		+6.0
0.005	5-6	5-6	6.0	4.6	5.0	6.6	6.4
0.001		6.2	6.4	5.4	5-6	6.6	6.2
0.0005	6.6	6.4	6-7	5.6	+6.0	6.6	6.6
0.0001	6.6	6.6	6-7	6.2	6.8	6.6	6.6
0.00005	6.6	6.8	6-7	6.4			6.6
0.00001	6.6	6.8	6-7	6.8			6.6
0.000005	6.6	6.8	6-7				6.6
Peptone 1 per cent	6.8-7.0						

the union of weak bases and a strong acid; and in an aqueous solution the bases formed tend to be undissociated or insoluble. Such solutions have an acid reaction due to the presence of an excess of H-ions. Thus in the stronger concentrations these salt solutions had a visible inorganic precipitate and even in the weak dilutions there was always a portion of the salt which was not in solution.

When solutions of these salts were added to peptone solution there was again a tendency for precipitates to be formed, this time of an organic nature and due to the fact that a peptone fraction was thrown out of solution. This precipitate was often greater than that occurring in peptone solutions which were brought to the same hydrogen ion concentration with HCl and

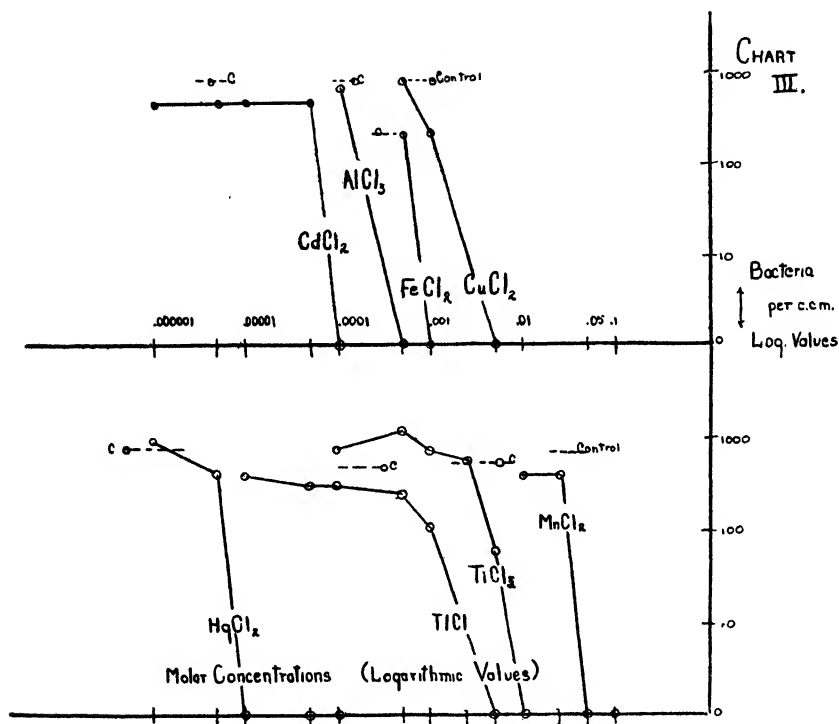


CHART 3. GROWTH OF *Bacterium coli* AS INFLUENCED BY VARIOUS SALTS OF THE HEAVY METALS

consequently must have been formed by action with the metal ion and not solely with the H-ions formed in hydrolysis. The work of many investigators tends to show that heavy metals which precipitate proteins, peptone or amino acids are themselves precipitated in so doing. In the formation of both of these precipitates some of the salt passes from the solution and we do not know the exact concentration of the final solution with which we were

working. In the preparation of tables and charts the molecular concentration as calculated in the preparation of the solutions is used but in any discussion of the results it must be remembered that a substantially smaller amount of salt was actually present in solution in the culture tube. Future work can define the toxicity points more closely by chemical analysis of the filtrates. The

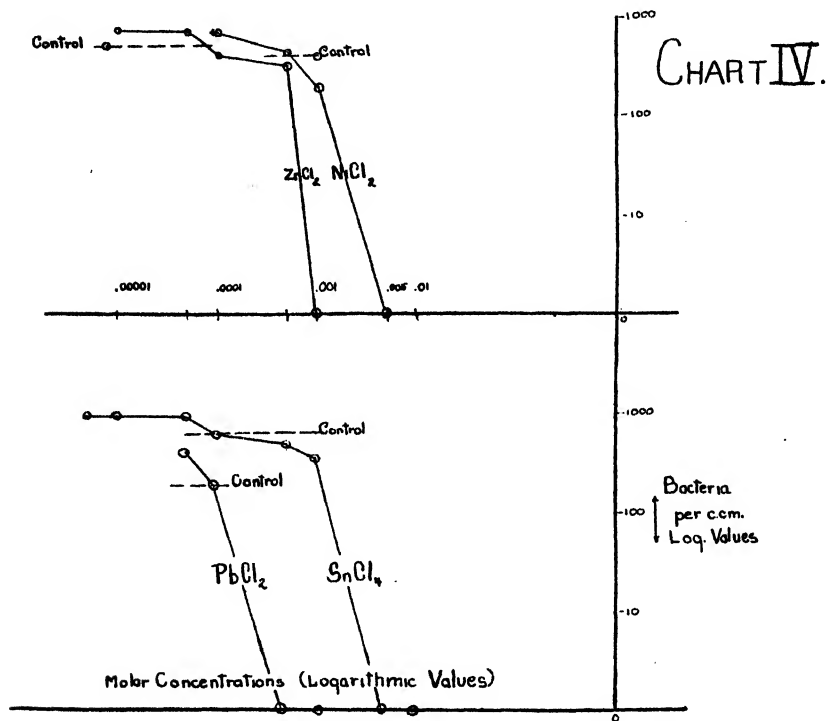


CHART 4. GROWTH OF *Bacterium coli* AS INFLUENCED BY VARIOUS SALTS OF THE HEAVY METALS

salts varied in the amount of precipitate formed in peptone solution. $SnCl_4$ caused the most marked precipitate of peptone solution per unit volume of salt, with $CeCl_3$ and $FeCl_3$ next, $AlCl_3$ and $BaCl_2$ third, $HgCl_2$, $PbCl_2$ and $TiCl_3$ fourth, and $CdCl_2$, $CuCl_2$, $FeCl_2$ and $ZnCl_2$ fifth, $CoCl_2$, $MnCl_2$, $NiCl_2$ and $TiCl$ did not give appreciable precipitates.

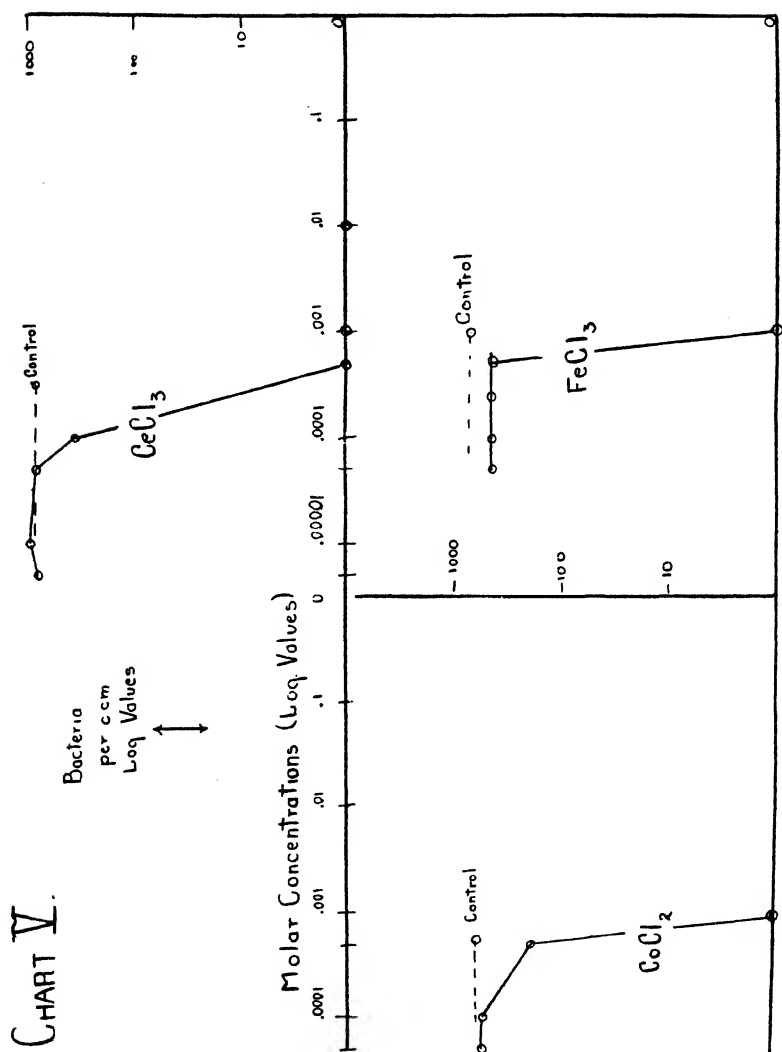
CHART 5. GROWTH OF *Bacterium coli* AS INFLUENCED BY VARIOUS SALTS OF THE HEAVY METALS

Table 4 gives the reaction after incubation and it will be noted that, as with the less toxic salts, where growth occurs the pH increases to between pH 8.0 and pH 9.0.

The varying toxicity points of the salts of group II, as determined at the end of a three days incubation period, are shown in table 5 and charts 3, 4 and 5. (Although its toxicity is notice-

TABLE 4

Hydrogen ion concentration after ten days incubation period. Salts of the heavy metals. pH readings (averaged)

CONCENTRATION	AlCl ₃	CdCl ₂	CeCl ₃	CoCl ₂	CuCl ₂	FeCl ₂	FeCl ₃
0.01 M			6.0				2.0
0.005				-5.0	4-5	4-5	
0.001	4-5		6.4	6.8	8.6	5-6	4-5
0.0005	5.4		6.5	8.4	8.7	7.1	8.3
0.0001	7.8	6.6	7.6	8.7	8.7	6.8	8.8
0.00005		8.9	8.6	8.7		6.8	8.8
0.00001		8.9	8.6			7.2	
0.000005		8.9	8.6				
CONCENTRATION	HgCl ₂	NiCl ₂	PbCl ₂	SnCl ₄	TiCl ₃	TiCl	ZnCl ₂
0.01 M		4-5		4.4	4.7		6.0
0.005		7.0	6.2	4.6	8.4	6.5	
0.001		8.0	6.5	8.4	8.6	6.2	6.4
0.0005	6.2	8.6	6.7	8.8	8.7	7.2	7.6
0.0001	6.6	8.8	8.5	8.6	8.7	7.6	7.8
0.00005	6.8	8.8	8.6	8.7		7.9	8.4
0.00001	7.0	8.8		8.7		8.6	8.7
0.000005	8.8	8.9		8.7			
0.000001	8.8						
Peptone 1 per cent	7.8						

ably less, manganese is included for convenience in charting.) Seven of the salts show stimulation of growth to an extent above that of the control, namely, cerium, nickel, lead, mercuric, stannic, titanium and zinc chlorides.

It seemed especially surprising that so toxic a compound as mercury should show this stimulation. The toxicity point was therefore checked by sterility tests made by streaking agar slants

with a loop of the culture. The lower dilutions did not show opalescence or any phenomenon which would interfere with the turbidity readings. No contaminations were found. The growth stimulation was not uniformly noted in the first days of incubation. It was most marked in the observations made after the ten day incubation period. The pH readings also testified

TABLE 5

*Average of readings for three days incubation period. Salts of the heavy metals.
Million bacteria per cubic centimeter*

CONCENTRATION	AlCl ₃	CdCl ₂	CeCl ₃	CoCl ₂	CuCl ₂	FeCl ₂	FeCl ₃
0.01 M			0				
0.005			0		0	0	
0.001			0	0	200	0	0
0.0005	0		0	200	700	200	550
0.0001	550	0	350	550			550
0.00005		450	700	550			550
0.00001		450	900				
0.000005		450	700				
Control	700	700	700	700	700	200	700

CONCENTRATION	HgCl ₂	NiCl ₂	PbCl ₂	SnCl ₄	TiCl ₃	TiCl	ZnCl ₂
0.01 M				0	0		0
0.005		0	0	0	60	0	
0.001		200	0	350	700	110	0
0.0005	0	450	0	500	1200	260	310
0.0001	0	700	300	600	700	330	400
0.00005	0	700	400	950		330	700
0.00001	0	700		950		430	700
0.000005	430	700		950			
0.000001	960						
Control	820	400	300	660	550	500	540

to increased growth for the peptone pH average was 8.3 and the average of the readings for the mercury solutions where increased growth occurs was 8.8 (see table 4).

The following experiment was undertaken to determine how far inhibition of growth by the salts of the heavy metals was due to the metallic ions themselves or to the hydrogen ion concentration.

Solutions of varying hydrogen ion concentrations were prepared by adding approximately $N/10$ HCl to the 1 per cent peptone solution. The hydrogen ion content was determined, as before, by the use of indicators and observations were made before and after sterilization. The peptone solution was sterilized in 5 cc. amounts and inoculated in the usual manner. Turbidity readings were made after varying incubation periods. The range of acidity unfavorable to this organism varied from pH 5.0 to pH 4.6.

An examination of tables 3 and 5 shows that for most salts of the heavy metals the pH is well beyond this unfavorable range in the highest dilutions at which growth is inhibited. In the case of three of the salts the free hydrogen ions may prove to be an important factor in toxicity.

	NO GROWTH	pH	GROWTH	pH
SnCl_4	0.005 M	4.6	0.001 M	5.4
FeCl_3	0.001	4.0-5.0	0.0005	6.0
AlCl_3	0.0005	4.0-5.0	0.0001	6.6

In these three salts growth occurred as soon as the dilution was used whose pH was favorable.

SUMMARY AND DISCUSSION

When grown in fluid culture media, bacteria produce a turbidity which can be used to estimate the number of bacteria per cubic centimeter. In these studies a 1 per cent peptone solution was used as a basic medium. Salts dissolved in the culture solution exerted an influence on the growth of *Bacterium coli* depending on the specific salt and the concentration in which it was added. There was some slight variation in effect at different incubation periods.

Each salt was so toxic at some concentration as to inhibit growth completely. The accompany table (table 6) shows the salt concentrations which thus limited bacterial growth. The group of the less toxic salts (termed group I in the preceding pages) includes the salts of the alkali metals and of the alkaline

earth metals. The toxic salts, or group II, consist of the salts of the heavy metals. It is seen that the salts of group I give neutral solutions and that, owing to hydrolysis, the salts of group II yield solutions with an acid reaction.

The results as to toxicity confirm, in general, those reported by Matthews (1904) for *Fundulus*, Woodruff and Bunzel (1909) for *Paramoecium* and Eisenberg (1916) for bacteria. The results of Eisenberg and the author in regard to relative toxicity parallel each other to a great extent. In 11 cases Eisenberg finds a

TABLE 6

Salt concentrations which limit bacterial growth. Incubation period, three days.
Molar concentrations

SALT	NO GROWTH	GROWTH	SALT	NO GROWTH	GROWTH
HgCl ₂	0.00001	0.000005	MnCl ₂	0.05	0.025
CdCl ₂	0.0001	0.00005	BaCl ₂	0.25	0.1
CeCl ₃	0.0005	0.0001	CaCl ₂	0.5	0.25
AlCl ₃	0.0005	0.0001	MgCl ₂	0.5	0.25
PbCl ₂	0.0005	0.0001	SrCl ₂	1.0	0.25
CoCl ₂	0.0005	0.0001			
FeCl ₂	0.001	0.0005	LiCl.....	0.75	0.5
FeCl ₃	0.001	0.0005	NH ₄ Cl.....	1.0	0.75
CuCl ₂	0.001	0.0005	NaCl.....	2.0	1.0
ZnCl ₂	0.001	0.0005	KCl.....	2.0	1.0
NiCl ₂	0.005	0.001			
SnCl ₄	0.005	0.001			
TiCl.....	0.005	0.001			
TiCl ₃	0.01	0.0025			

higher molar concentration necessary for toxicity, in 4 cases the toxic concentrations are lower and in 6 the toxic concentrations are about the same as those of the author.

When the toxic concentrations are calculated in terms of molar solutions, the different salts may be arranged in a series of ascending toxicities as in table 7. The table shows, for each of the four workers (Matthews, Woodruff and Bunzel, Eisenberg and the author) a non-toxic and a toxic group of salts. The salts contained in each group are nearly identical, but in Matthews' work

the AlCl_3 and the FeCl_3 fall in the non-toxic group. Matthews found a great difference in the action of the bi-valent FeCl_2 and the tri-valent FeCl_3 which Eisenberg did not observe, and which did not appear in the present study.

TABLE 7
Relative toxicities according to four observers

MATTHEWS	WOODRUFF AND BUNZEL	EISENBERG	AUTHOR
SrCl_2	KCl	NaCl	NaCl , KCl
BaCl_2	CaCl_2	KCl	NH_4Cl
MgCl_2	SrCl_2	NH_4Cl	LiCl
AlCl_3		LiCl	
NH_4Cl	MgCl_2	MgCl_2	SrCl_2
KCl		SrCl_2	CaCl_2 , MgCl_2
CaCl_2	MnCl_2	CaCl_2	BaCl_2
MnCl_2		BaCl_2	MnCl_2
LiCl	CoCl_2	MnCl_2	
FeCl_2	CuCl_2		TiCl_3
		HCl	
CoCl_2	CdCl_2		TiCl , NiCl_2 , SnCl_4
NiCl_2	NiCl_2	CeCl_3	
		CrCl	ZnCl_2 , CuCl_2 , FeCl_2 , FeCl_3
ZnCl_2	PbCl_2	FeCl_2	
		FeCl_3	AlCl_3 , CeCl_3 , PbCl_2 , CoCl_2
HCl		ZnCl_2	
CdCl_2	FeCl_3	ThCl_4	CdCl_2
AuCl_3			
CuCl_2	HgCl_2	AlCl_3	HgCl_2
		CuCl_2	
FeCl_3		TiCl_4	
		NiCl_2 , TiCl	
		CdCl_2	
		PbCl_2	
		CoCl_2	
		AuCl_3	
		PtCl_4	
		HgCl_2	

In 15 of the 23 chlorides studied a concentration was found which stimulated growth, as indicated by the production of a turbidity greater than that in the control solution to which no salt had been added (table 8). The stimulating salts included

not only K, Na, NH_3 , Li, Sr, Mg, Ca and Ba but such toxic salts as those of Ti, Sn, Ni, Pb, Co and Hg. The stimulating concentrations for the latter were, of course, exceedingly low, (0.00005 molar in the case of Pb, 0.00001 molar in the case of Ce, 0.000001 molar in the case of Hg) while with K and Na a 0.25 molar concentration was stimulating. It is very possible that stimulating concentrations of the other 8 salts could have been established by more exhaustive study.

TABLE 8

Molar concentrations which stimulate bacterial growth. Three days incubation

SALT	CONCENTRATION	SALT	CONCENTRATION
NaCl.....	0.25 M	TiCl ₃	0.0005 M
KCl.....	0.25	NiCl ₂	0.0001—0.00005
NH ₄ Cl.....	0.25	PbCl ₂	0.00005
LiCl.....	0.125—0.025*	SnCl ₄	0.00005—0.000005
		ZnCl ₂	0.00005—0.00001
CaCl ₂	0.05	CeCl ₃	0.00001*
MgCl ₂	0.05	HgCl ₂	0.000001*
SrCl ₂	0.025		
BaCl ₂	0.05		

* Growth stimulation slight.

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LE BACTERIOPHAGE DE D'HERELLE: SES APPLICATIONS THERAPEUTIQUES

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Depuis l'époque où d'Herelle présentait à l'Académie des sciences de Paris ses premières communications (1917), des expérimentateurs nombreux se sont mis à l'étude du phénomène qu'il a décrit. Nous le résumons en quelques mots: le filtrat d'un bouillon ordinaire dans lequel on met à incuber des selles de convalescent de dysentérie bacillaire ou de typhoïde présente la propriété de lyser une culture jeune de bacilles dysentériques ou typhiques.

D'Herelle a très soigneusement étudié le fait qu'il avait mis en évidence, il en a abordé tous les aspects, a su construire une théorie de l'immunité par le bactériophage, apercevoir les applications thérapeutiques étendues qu'on pouvait en tirer. Nous allons, dans cet article, exposer, son hypothèse sur la nature du principe bactériophage et sur quelles expériences il l'appuie. Nous rappellerons que d'autres hypothèses ont été émises, mais, sans prétendre à être complets.

Il est auparavant un point historique curieux à élucider. En parcourant systématiquement les revues scientifiques, on trouve que certains expérimentateurs ont vus le phénomène avant lui, beaucoup ont eu entre les mains du bactériophage. Dans le "Traité de Microbiologie" de Duclaux on lit cette phrase "Rappelons les curieuses expériences de Hankin sur les eaux de la Jumma et du Gange, où on voit une eau courante être aussi bactéricide qu'une solution de sublimé et perdre ce pouvoir après une courte ébullition sans qu'aucun précipité, sans que rien d'apparent, explique ce changement de propriétés. . . ."(1898-1901). Hankin dans les Annales de l'Institut

Pasteur (1896) insiste sur cette action bactericide qui lui semble difficilement explicable.

Elle est à mettre au compte de la présence du bactériophage. Dans son livre "Le Bacteriophage" (1921), d'Herelle signale un autre fait du même ordre. Eliava, chargé d'examiner l'eau de la rivière Koura qui passe à Tiflis, y constate le phénomène suivant; s'il additionnait l'eau suspecte de peptone, après quelques heures d'incubation, on constatait la présence de très nombreux vibrions de forme normale; une douzaine d'heures plus tard, toute trace de ceux-ci avait disparu. Le phénomène resta pour lui inexplicable tant qu'il n'eut pas connaissance des communications concernant le bactériophage. En 1916 et 1917, Gildemeister écrit de longs articles dans le "Centralblatt für Bakteriologie" (1916-1917) pour décrire des figures de transformation du bacille dysentérique, du bacille typhique et du colibacille. Il étudie très complètement les colonies atypiques qu'il rencontre, et essaie de les classer. Le passage de colonies de formes atypiques à la forme typique le frappe; il insiste sur le fait que c'est au sortir de l'organisme qu'on rencontre de telles colonies. Les articles sont illustrés de photographies qui montrent que, sans aucun doute, Gildemeister a manié des colonies parasitées par du bactériophage.

Plus récemment Howard a signalé à la Société de Biologie de Paris (1920) l'action lytique de certains sérums de typhiques: C'est là un phénomène que nous avons retrouvé et signalé (1922). Nous l'attribuons, pour notre compte, au bactériophage.

On voit, par ces quelques exemples, que le phénomène de la lyse en série de certaines bactéries, que les modifications apportées par le bactériophage dans l'aspect des colonies, ont été aperçus par les expérimentateurs. D'Herelle a retrouvé de semblables faits quand, à l'occasion d'une observation fortuite dans l'étude de la cocco-bacillose des sauterelles, il constata un arrêt inexplicable de ses cultures. Il y revint plus tard, envisagea de nombreuses hypothèses avant de s'arrêter à celle qui lui parut concorder étroitement avec ses expériences.

Un grand nombre de bactériologistes avaient donc vu "le bactériophage," mais c'est d'Herelle qui l'a "découvert," nous

semble-t-il. On a beaucoup parlé, d'autre part, d'un phénomène décrit par Twort en 1915. Bordet, en particulier, (1921) veut y voir la lyse transmissible en série et il s'est fondé là-dessus pour contester à d'Herelle la priorité de sa découverte. Or la description donnée par Twort n'a que d'assez lointains rapports avec le bactériophage. Nous ne saurions entrer dans le détail d'une discussion, mais il nous paraît probable que le phénomène de Twort et le phénomène de d'Herelle ne sont pas identiques.

Qu'est ce pour d'Herelle que le bacteriophage? Reproduisons la définition qu'il en donne lui-même dans son livre (*Le Bactériophage*, p. 10).

C'est un petit être vivant, sans aucune présomption sur la règne auquel il peut être rattaché. Est-ce une bactérie, un protozoaire, un champignon? Je l'ignore, ses dimensions sont trop réduites pour qu'on puisse résoudre la question par l'observation directe au moyen des instruments optiques actuels. C'est une particule qui se développe aux dépens de la substance des bactéries, capable par conséquent d'assimilation, et qui est cultivable en série *in vitro* sous sa forme filtrante.

Les expériences qu'il apporte à l'appui de sa théorie sont nombreuses, elles semblent exactes. Il est curieux de constater qu'un certain nombre de savants, ont émis d'autres hypothèses, sans soumettre à une vérification préalable maintes expériences fondamentales de d'Herelle.

Celui-ci base sa théorie d'un ultra-microbe sur quatre ordres de faits:

1. Transmissibilité indéfinie en série.
2. Numération possible des éléments bactériophages.
3. Adaptation du bactériophage à certains corps chimiques.
4. Polyvalence du bactériophage.

Examinons-les séparément.

On peut transmettre en série, indéfiniment, le bactériophage. A partir d'un premier tube où la lyse s'est produite on transporte après chauffage ou filtration pour éviter les cultures secondaires, une goutte capillaire dans un second tube contenant une culture jeune du microbe lysable. La lyse se produit. Elle peut être

répétée aussi souvent qu'il plaira. C'est la reprise des expériences de Pasteur en vue d'obtenir des cultures pures et la preuve que le bactériophage est un principe apte à se reproduire.

La numération du bactériophage peut se faire soit en bouillon, soit sur gélose inclinée. En bouillon, par la méthode des dilutions, parmi les plus élevées, on note certains tubes où la lyse ne se fait pas, d'autres où elle est encore positive. Les premiers ne contenaient plus de bactériophage, les autres en avaient conservé un ou deux éléments susceptibles d'ailleurs de déclencher une lyse totale et de fournir, au titrage, un produit d'activité égale à l'activité initiale: autre preuve de la reproduction du bactériophage. Nous avons montré (1912) que la numération sur gélose inclinée est plus sensible que la méthode précédente. La présence du bactériophage se traduit par des "plages claires" petits trous restés stériles au milieu de la nappe de culture homogène. Ce seraient autant de véritables colonies isolées de bactériophage et le fait qu'on puisse numérer un "principe," rend lui faire accorder une forme organisée.

Le bactériophage peut s'adapter à certains antiseptiques, la glycérine en particulier. En soumettant à l'évaporation une émulsion glycérinée de bactériophage, de manière à augmenter la concentration on retrouve à la fin de l'expérience le lysat vivant. Il périrait inmanquablement dans une solution de glycérine aussi concentrée où il serait transporté d'emblée. On connaît des cas nombreux où les microbes s'accoutument aux antiseptiques: "l'accoutumance est l'apanage exclusif des êtres vivants."

Le bactériophage est un: on entend par là qu'il possède une virulence vis-à-vis de nombreuses bactéries. Quand on isole un bactériophage primitivement actif pour le coli-bacille, on peut l'amener à lyser le bacille typhique par exemple, il n'est pas invraisemblable que des passages suffisamment répétés puissent l'amener à lyser n'importe quelle bactérie.

Tous ces arguments constituent à l'hypothèse de d'Herelle un solide appui. Que lui objecte-t-on? En jetant un regard en arrière sur la masse des documents publiés, rien de probant! Personne n'a versé au débat un fait précis, une expérience décisive qui renverse les conclusions de d'Herelle.

Nous laisserons de côté les théories de Kabeshima et de Salimben basées sur les expériences ou des constatations qui ne sont pas à l'abri des critiques. Bordet injectant dans le péritoine d'un cobaye du coli-bacille retire quelques heures après un exsudat contenant du bactériophage. Il échafaude toute une théorie: celle de l'autolyse microbienne transmissible, bien que les résultats des cette expérience soient irréguliers et que peu d'auteurs aient pu la répéter. La raison en est dans ce double fait: c'est le bactériophage préexistant dans l'intestin du cobaye qu'on retire à la faveur de la lésion minime produite par l'inoculation intrapéritonéale et au surplus la présence de ce bactériophage est irrégulière.

Lisbonne et Carrère ont émis successivement deux théories. Il ont d'abord fait provenir le bactériophage des leucocytes: cette première hypothèse fut infirmée par d'Herelle, (1922a, 1922b). Récemment, ils affirmèrent que le simple jeu d'un antagonisme microbien suffisait pour faire apparaître la lyse en série (1922). En mélangeant du *Bacterium coli* et du bacille de Shiga, en filtrant les cultures obtenues et en faisant un certain nombre de passages, on verrait la lyse se produire. Nous croyons avoir montré qu'il étaient partis de cultures impures, de colonies modifiées parasitées par du bactériophage (1922).

Ces discussions sur la nature du bactériophage présentent un intérêt biologique considérable, mais un autre aspect de la question n'a pas été étudié avec l'ampleur qu'il mérite: c'est le chapitre des applications thérapeutiques, à peine ouvert à l'heure actuelle.

Dans sa première communication d'Herelle annonçait que son principe était capable de préserver le lapin contre une inoculation mortelle de bacille de Shiga. Ces premières expériences furent complétées par d'autres sur la typhose aviaire, le barbone. De la pathologie animale il est passé la pathologie humaine, a traité des dysentériques à l'aide d'ingestions de lysat. Les applications sont limitées, pour le moment, par le nombre encore peu élevé des souches actives qu'on possède: anti-dysentériques et paradysentériques, anti-typhiques et anti-paratyphiques, anti-staphylococcoque, anti-entérococcique, anti-bacterium-coli, anti-bacilli pestueux, anti-vibron cholérique.

Résumons les résultats apportés par divers expérimentateurs et par nous-mêmes dans les diverses affections traitées. Une importante remarque préjudicielle s'impose: comme le bactériophage représente une médication nouvelle, les statistiques ne sont pas encore assez fournies pour décider s'il est le remède spécifique de la dysentérie ou de telle autre maladie. Nous en sommes à la période d'essai. Une précaution préalable consistera dans l'isolement du microbe en cause et sa mise en présence du bactériophage, qui le lysera ou non *in vitro*. On reconnaît par là les cas favorables, de ceux qui ne sont pas justiciable du traitement. Le mode d'administration le meilleur et la fixation des doses sont encore imparfaitement au point. Dans nos essais personnels nous avons tenté d'établir une posologie mais celle-ci varie en fonction de la virulence des souches.

LE BACTÉRIOPHAGE DANS LA DYSENTÉRIE BACILLAIRE

D'Herelle, dans son ouvrage (*Le bactériophage*, p. 211), rapporte sept cas de dysentérie traités par la seule ingestion de lysat. La plupart des malades étaient gravement atteints. 24 à 48 heures après l'absorption de bactériophage par la bouche, l'état général s'améliore, les selles sanglantes disparaissent deviennent moulées et la convalescence s'établit dans les 2-3 jours. Ce sont là des résultats impressionnants. Il paraît que Otto et Munter n'auraient pu les confirmer. Ne connaissant pas leurs observations nous n'en pouvons rien dire.

LE BACTÉRIOPHAGE DANS LA FIÈVRE TYPHOÏDE

Au cours d'une épidémie de fièvre typhoïde nous avons pu faire les premiers essais de traitement à l'aide du bactériophage. Le 13 janvier 1922 nous apportons à la Société de Biologie les résultats suivants:

Deux cas chez l'adulte: formes ataxo-adiynamiques; atteinte sévère du myocarde: (a) ingestion de lysat au 8^{ième} jour: crise de sueurs dans les deux heures; le surlendemain apyrexie d'une durée de 48 heures; (b) ingestion au 20^{ième} jour: pas de modification. Dans les deux cas, reprise et mort.

Deux cas, de typhoïde chez l'adulte: formes ordinaires (a) ingestion au 18^{ième} jour défervescence en lysis à partir du 20^{ième} jour. (b) ingestion au 9^{ième} jour, lysis à partir du 11^{ième} jour. Crise de sueur dans les deux cas deux heures après l'ingestion. Convalescence.

Un cas de typhoïde chez l'enfant: forme grave. Bactériophage par les deux voies au 20^{ième} jour: Le surlendemain, l'apyrexie, nette et durable, succède aux températures élevées (un autre cas à association anaérobie parable n'a pas été influencé).

Deux cas de para-typhoïde B chez l'enfant: (a) Etat grave, bactériophage par les deux voies au 9^{ième} jour, apyrexie nette et durable le lendemain, (b) forme ordinaire, administration au 23^{ième} jour; apyrexie à partir du surlendemain.

Le résultats de ces 7 cas ne sont pas encore probants. Il semble bien que le bactériophage ait exercé une action favorable, mais nous pensons que le échecs sont dus, partie à une intervention trop tardive, partie à la faiblesse des doses administrées. Jusque là nous avons donné deux centimètres cubes au plus. Deux enfants atteints d'infection sévère sont traités par 5 cc. per os et 1 cc. sous la peau, l'un au 10^{ième} jour, l'autre au 14^{ième} jour. Dans les deux cas, obnubilation et température élevée font place dans les 48 heures à la défervescence durable et à une euphorie marquée.

Un 3^e cas, traité énergiquement chez un sujet de 24 ans, nous a procuré au 18^{ième} jour, en 3 jours, une défervescence nette et durable avec euphorie marquée (bien qu'au prix d'une réaction thermique un peu vive).

Si ces essais n'apparaissent pas comme décisifs, ils révèlent cependant une coïncidence impressionnante entre l'administration de lysat et une amélioration évidente. La réunion d'un grand nombre de cas apportera, croyons-nous, la preuve de l'efficacité du bactériophage dans le traitement de cette infection.

INFECTION À STAPHYLOCOQUES

Bruynoghe et Maisin ont été les premiers à appliquer le bactériophage dans les infections à staphylocoques (1921). Il se sont adressés à des malades atteints, soit de furonculose,

soit d'anthrax. Aussi près que possible de la région malade ils injectent 0.5 cc. à 2 cc. de lysat. L'empâtement diminue rapidement et les lésions disparaissent en 24 ou 48 heures.

Gratia (1922) a publié les résultats obtenus par lui. Dans les cas d'abcès et de furoncles le processus de guérison est notablement accéléré par l'administration de lysat. Le pus est évacué rapidement parfois même se résorbe sans laisser de cicatrices. Nous avons, de notre côté, tenté des essais qui seront publiés quand nous estimerons avoir réuni un nombre suffisant d'observations pour pouvoir fixer la posologie et le mode d'administration de ce mode de traitement. D'ores et déjà nous indiquerons qu'au lieu d'un bactériophage anti-staphylocoque doré unique, nous utilisons un mélange de bactériophages spécialement et séparément exaltés: anti-staphylocoque-doré, anti-staphylocoque-blanc et anti-entérocoque. Nous ménageant ainsi plus de chances d'arriver à un résultat favorable au cas d'une infection polymicrobienne éventuelle. Nous prenons toujours soin (ici encore) de faire un essai de lyse in vitro du microbe isolé. Cette précaution nous a d'ailleurs conduits à la constatation que les germes isolés d'infections chroniques sont fréquemment résistants à la lyse.

Nos résultats, d'une manière générale, concordent avec ceux de Bruyngoghe et de Gratia.

En somme, si les travaux accumulés n'ont pas indiscutablement mis en lumière la nature du bactériophage, il n'en importe pas moins de s'engager sans tarder dans la voie, qui promet d'être fertile, des applications thérapeutiques étendues et variées.

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ON THE VARIATION OF BACTERIUM COLI

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INTRODUCTION

Before the time of Koch bacteria were supposed to be capable of a very great variation. But the plating method showed that the supposed variants were usually contaminating microorganisms. As so often happens, the new discoveries and the theories based on them were over-emphasized and bacteriologists went to the other extreme. Of course a great many articles were written about bacterial variation, but they were never taken into consideration. Even the striking discovery of the *Bacterium coli mutabile* by Neisser and Massini (1907) did not attract any very general interest. Everybody who has read, for example, the modern literature on the types of pneumococci and meningococci, etc., must admit that there is very little talk about variation of the types. As far as I can understand, the types are supposed to be constant. A pathogenic type can change its virulence, but it is not to be confused with an apathogenic one.

In the case of pneumococcus the distinction between the types is made only by the agglutination test. They do not differ morphologically or culturally. It is then justifiable to ask if it is not possible that one type can change into another by losing or acquiring agglutinability, erroneously regarded as specific. As a matter of fact this is already shown to be the case with *Bacterium coli*. Baerthlein (1912) obtained from a pure culture of *Bacterium coli* two types, one agglutinable not only by its own antiserum, but also in high dilution by the antiserum of the other type, which, on the contrary, was very slightly agglutinable by either serum. And neither of these types was constant, one

after a while passing over into the other. These discoveries have recently been partly confirmed by Bordet and Ciuca (1920) and Gratia (1922) in their works on the Twort-d'Hérelle phenomenon.

Certainly the problem is of interest and involves questions of great importance. One may ask: does a variation of a non-pathogenic type into a pathogenic one often happen, and under what conditions? Does the isolation of the patient really compensate for the effort and the expense under such circumstances? It is obvious that the answers to these questions will be very different in the case of different bacteria. We know that a satisfactory isolation against *Spirochaeta pallida* ought to check syphilis, but we are not sure of the result in the case of diphtheria and still less in pneumonia. There is justification for the assumption that an apathogenic pneumococcus can change into a pathogenic one and give rise to an epidemic or only to some single cases. It is also possible that this epidemic may disappear, not only because the individuals still living have acquired immunity, but also because the microorganisms are inclined in time to change their type. Topley's (1921) remarkable observations in spontaneous epidemics among white mice point in this direction. He showed that the microorganisms and the living animals established a *modus vivendi* and the epidemic died, but when new animals were mixed with the old a new epidemic occurred, affecting not only the new but also the old "immune" individuals.

The above-mentioned facts are sufficient, I think, to demonstrate that the variation of bacteria is well worth more general interest. Fortunately the recent discovery of the Twort-d'Hérelle phenomenon seems to give these studies new life.

In the investigations on bacterial variation, related in the following pages *Bacterium coli* is used as an object for several reasons. This bacterium has been worked with on these lines by many previous authors and it seems to be subject to variation in a specially high degree; it is easy to cultivate and harmless to handle.

1. Isolation of the types

An old laboratory culture of *Bacterium coli* was seeded in broth and left for a fortnight and then plated. The plates showed two kinds of colonies, which were called D (dull) and S (shining). The D-colonies were large, non-transparent, yellowish, with an uneven stripy border and a dull slightly corrugated surface; the S-colonies were small, transparent, bluish, sharp-bordered and showed an even shining surface. The populations of the cultures also differed morphologically, the D-type consisting mostly of long slender individuals and the S-type of short clumsy ones. On fishing, the D-colonies turned out to be dry and cracky, the S-colonies, on the contrary, moist and of a paste-like consistency. This difference is demonstrated in figure 4. Both types possess the general properities of *Bact. coli* with regard to morphology, the sugar fermentation tests and the indol production test. They are also agglutinated by several coli sera, as will be mentioned later on. The D-type is not motile; the S-type on the other hand is very motile. In a twenty-four-hour-old broth-culture (pH 6.8) the D-type produces a supernatant film and a sediment on the bottom of the tube; the broth between the film and the sediment is clear. The tubes seeded with coli S show a homogeneous turbidity and a whitish band at the level of the fluid surface, soon developing to a film. Later a sediment is to be seen also in these tubes. But a difference persists for weeks between the broth-cultures of the two types. The broth never becomes clear in the S-tubes and the sediment is cloudy with an uneven surface. The sediment in the D-cultures has a smooth surface just like sand in the bottom of a glass of water (fig. 10). It is evident that the type S corresponds to one of the types observed with *Bact. coli* by Gratia and described as type R. He found on old agar slants in the dull film small vitreous colonies containing a pure culture of a type differing greatly from the organisms of the original strain. This new type was very resistant against the lytic agent, more virulent and less phagocytal than the original type, and had a lively motility. On the other hand he observed a non-motile sensitive and aviru-

lent type, that evidently is identical with our type D. Baerthlein also talks about opaque (trüb) and non-opaque (hell) colonies.

After repeated plating isolation was made *ad modum* Burri in order to obtain cultures of as great a purity as possible. I am of course aware of the fact that no certain method of obtaining single cell cultures exists. It is possible however, to keep the cultures pure for months by daily plating and their general behavior in the experiments affords sufficient evidence that these first cultures can be accepted as derived from a single cell or at least from a couple of identical cells.

EXPERIMENTAL

Experiment 1. A loopful was taken from an eighteen hours slanted agar culture of each type and spread out in a test-tube containing 5 cc. plain broth of a hydrogen concentration of pH 6.8. The bacteria of the D-culture were very difficult to spread. At last, however, a homogeneous suspension seemed to be obtained not only of the S-type, which easily mixed with the broth, but also of the D-type.

From these two suspensions two equal series of tubes containing broth of decreasing hydrogen concentration were seeded, each tube with 0.5 cc. Kept at room-temperature (18°C.) for eighteen hours a loopful was removed from the culture and thoroughly mixed with 5 cc. plain broth of the same above-mentioned hydrogen concentration. From each of these dilutions in turn a loopful was spread on agar-plates, incubated for eighteen hours, and then examined. The number of the colonies and the pH values of the corresponding tubes are indicated in table 1. This first experiment was carried out on February 13 to 15. Afterwards samples were removed and treated in the same way on February 18, February 25, March 5 and March 14 in order to study the supposed appearance of variants and their behavior. The results of these platings as to the numbers of colonies are recorded in tables 2 to 5. By examining the first series of plates a striking lack of parallelism between the size and number of the D-colonies was the most outstanding feature. The D-colonies were several times larger

TABLE 1
February 15

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH.....	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S.....	0	1	71	67	59	84	60	91
Type D.....	0	0	19	22	26	19	21	25

TABLE 2
February 18

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH.....	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S.....	0	190	1200	1400	1400	1200	1100	1300
Type D.....	0	5	43	46	110	90	84	115

TABLE 3
February 25

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH.....	4	5.1	5.3	6.6	7.2	7.6	8	8.4
Type S.....	0	500	500	600	800	500	500	600
Type D.....	0	100	78	80	110	150	250	200

TABLE 4
March 5

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH.....	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S.....	0	500	150	200	150	200	200	200
Type D.....	0	31	150	150	250	250	150	250

TABLE 5
March 14

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH.....	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S.....	0	150	110	95	120	90	140	63
Type D.....	0	120	150	200	225	65	110	200

in size than the S-colonies but the latter were much more numerous. Granted that the organisms are of the same shape and that the difference is not due to an excess of culture-medium in the D-plates, this fact must be regarded as a contradiction. That the D-colonies really grow more rapidly than the S-colonies on agar-plates was shown by making plates containing the same numbers of colonies spread regularly over the plates. Furthermore, the D-microorganisms certainly differ in size from the S-microorganisms, but this could not be accepted as a sufficient explanation. In order to solve the question a new experiment was carried out.

Experiment 2. As it was necessary to obtain a bacterial suspension of as exactly known a density as possible, a broth-culture eighteen hours old was thoroughly shaken for one-half hour, diluted and then mixed with a suspension of washed red blood-corpuses. Examination of a few samples now showed that it was very easy to get the S-microorganisms isolated but almost impossible to separate the D-bacteria sufficiently. Thus it was immediately clear that the great size of the D-colonies is due to this fact; they do not originate from single cells, but, at least mostly, from clusters of cells. This will also be recognized from the following experiment, revealing at the same time a new phenomenon.

Experiment 3. In order to obtain a good suspension and at the same time to study the effect of a most thorough shaking, a broth-culture of each of the two types was first shaken during five minutes and a sample removed and spread on agar, then left one-half hour and afterwards shaken again with glass-beads during one-half hour and an equal sample plated. Originally it was intended that the clusters should sink to the bottom, thus making it possible to obtain a homogeneous suspension in the fluid above. But the D-tubes showed already when examined macroscopically a poor suspension. Hence the second shaking was made. Plating now showed the remarkable fact that the D-colonies had decreased and the S-colonies increased in number. Thus the shaking with the glass-beads had caused an agglutination of the D-microorganism, which could be confirmed by

direct microscopical observation. On the other hand the S-bacteria had been separated in a higher degree by the second shaking than before. The details are indicated in table 6.

These findings are of importance in considering the above-mentioned plating experiments and the agglutination tests described below. They show that even when the colonies look as if they had originated from one single cell this is not at all certain, and that the plating method has only a relative value in counting the living microorganisms in a broth-culture, at least as far as *Bact. coli* is concerned.

Experiment 4. As is to be seen in tables 1 to 5, the limiting pH values on the acid side lie between pH 4 and pH 5.1. and seem to be the same for both types. In the first tube, of the highest hydrogen ion concentration, not only does no growth occur but the microorganisms are killed and this occurs, as a new

TABLE 6

TYPE	D	S
Numbers of colonies after the first shaking.....	42	76
Numbers of colonies after the second shaking.....	13	275

experiment showed, in thirty-six hours for the S-type and in twenty hours for the D-type. In the second tubes too at first many bacteria die and it is only after an interval of several hours that a part of them recover from the injuries and begin to multiply. This is shown by the fact that plates seeded with a loopful from control-tubes containing 5 cc. broth inoculated immediately before with one-half cc. of the original bacillary suspension show from five to ten colonies. In the second tubes the growth is delayed to a very considerable degree but rises in time to the same level as in the other tubes. The decrease of the living microorganisms in the cultures occurs earlier in the S-tubes and is more marked. It appears that the number of viable bacteria for long periods is fairly the same in both S-tubes and D-tubes with perhaps an increase in later tubes as shown in table 5. Considering the peculiarities mentioned in experiment 3 it is, however, necessary to be careful that this is

not due merely to a higher degree of suspension of the bacteria. On the other hand there appeared in the D-tubes a new phenomenon which, as we shall see later, is perhaps responsible for these figures. In order to obtain the limiting hydrogen concentrations with as great accuracy as possible two new series were seeded and examined in the same way as before, except that the plating was made twenty-four hours after the seeding of the tubes. The results are indicated in table 7. The method used for preparing the broth-tubes was the following. Two litres of broth are obtained by boiling 1 kgm. minced ox meat for one hour and adding 20 gram pepton Witte and 10 gram NaCl. The broth divided in small portions is adjusted by NaOH and HCl to the desired pH, filtered into tubes and sterilized. Afterwards the tubes are tested again and suitable sets taken for use. The test

TABLE 7

April 8

	NUMBER OF TUBE														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
pH.....	4.3	4.8	5.0	5.2	5.8	6.3	6.8	7.2	7.6	8.2	8.6	9.0	9.2	9.3	9.4
Type S.....	0	0	62	400	500	500	500	500	600	600	100	42	65	17	0
Type D.....	0	0	12	36	42	250	250	250	300	250	45	0	0	0	0

of the hydrogen ion concentration was made by the colorimetric method and the indicators used were those of Clark and Lubs, kindly provided by K. G. Dernby.

This experiment evidently confirms the above mentioned observation that a too high hydrogen concentration delays the growth of the type D more than the growth of the type S. Or in other words the "lag" is greater in type D. The "lag" is very well marked up to the D tube 6, while type S multiplies practically just as rapidly in tube 4 as in tube 8. On the alkaline side too, the type D is more sensitive. A pH value of 8.6 gives a marked "lag" and a pH of 9.0 kills the microorganisms, as samples removed some days later showed. On the other hand type S shows a great resistance, multiplying up to a pH value of 9.3; and control-tubes showed that a number of the seventeen colonies were capable of growth.

Experiment 5. The tubes were left for a month and then examined. Now it turned out that the "lag" was not the only injury that the high and the low hydrogen ion concentration caused. It may be true that the tubes at the ends of the series also got their chance and for a time developed as rapid a growth as the other tubes, as is indicated in the series indicated in tables 2 to 5, but a comparison of the sediment in the different cultures shows that the sum of growth is far less in these delayed tubes.

The sediment having been measured, the tubes were filtered and the filtrates tested for pH. The examination showed that the hydrogen ion concentration had decreased in the tubes on the left side and increased on the right side. The pH value was much alike in all tubes oscillating only between 6.8 and 7.2 approximately. The microörganisms evidently had readjusted the hydrogen ion concentration to their own benefit in a very remarkable way. An exception on the acid side was noted in tubes 1 to 3 of both series. This is quite natural for tubes 1 and 2 but surprising in the case of tube 3. The difference in hydrogen ion concentration between tube 3 and 4, slight in the non-seeded broth, was in the filtrates really striking, when we recollect that tube 3 also developed an appreciable growth. The remains of the cultures at the ends of the series were now seeded in plain broth and this experiment showed that the cultures of No. 3 were dead. Thus it seems as if the bacteria in these tubes died because they were unable to make a growth strong enough to readjust the medium to a hydrogen concentration necessary for a longer life. That the first series did not reveal this phenomenon may be due to the higher pH value (5.2) in the first tube capable of growth in these series.

No corresponding phenomenon was observed on the alkaline side where only the tubes without growth were unchanged.

In the preceding notes we have chiefly considered the numbers of colonies observed on the agar-plates seeded from the broth-cultures but these themselves show several features worth mentioning. I only record the findings in the series of April 8, since they do not differ from the observations made in the first experiments. The D-tubes 6, 7, 8, 9, 10, kept at room-tempera-

ture (18 cc.) for twenty-four hours, all show a sediment of the same thickness and a clear fluid above: in the eleventh tube no growth is visible; tubes 3, 4, 5 are slightly turbid but without sediment. The S-tubes 4, 5, 6, 7, 8, 9, 10 are very turbid and tubes 12, 13, 14 are slightly troubled, all without sediment; tube 3 shows a small sediment and a clear fluid. Apparently the D-cultures show the character of an S-culture and conversely in the tubes approaching the limit of the hydrogen concentration on the acid side, but both keep their character on the alkaline side. In the D-tube 11 the broth is clear and the growth invisible, since the sediment is not yet heavy enough to be recognized. Thus the behavior of a type of *Bact. coli* in a broth-culture is characteristic only at a fixed hydrogen ion concentration. Spread on agar the cultures show no difference. Evidently we are not dealing with any hereditary fixed modification.

After forty-eight hours a considerable sediment has developed in the S-tubes 4, 5, 6, 7, 8, 9, 10 and a less marked one in the third and eleventh. The other S-tubes and the D-tubes have practically the same appearance as before. Already during the first twenty-four hours a whitish band has appeared in the S-tubes adhering to the glass wall at the level of the fluid surface and from this band a supernatant film developed during the next day. At the same time that the whitish band became visible in the S-tubes the first traces of a supernatant film appeared in the D-tubes. The film reached in a short time a much stronger development in the D-tubes than in the S-tubes. My observations in this matter are not in absolute agreement with those of Gratia, who writes that his R-type (corresponding to my S-type) multiplies slowly and produces a film later. As is indicated by the above-mentioned experiments, it is, however, very difficult to judge which type really multiplies most quickly. In the following days the film sinks to the bottom and the cultures assume their definite features described above and demonstrated in figure 10.

DESCRIPTION OF THE VARIANTS

Shortly after the first definite isolation of the types both were seeded on agar-plates in such abundance that on the S-plates an uninterrupted film soon developed. On the plates of the D-type no confluence occurred. A month afterwards the D-plates showed a great many small secondary colonies (fig. 2), resembling transparent shining droplets. Evidently these secondary colonies correspond to those described by Massini with *B. coli-mutabile* and more elaborately dealt with by Nyberg (1912) under the name of "daughter colonies." Fishing from these colonies did not produce any other colonies than the common dull type, when streaks were made on agar-plates either directly or after dilution in broth. The S-plates (fig. 1), on the other hand, showed very few secondary colonies and of an entirely different character, having the form of rather big rings, growing up a little over the surface of the film. It was easy to obtain by fishing from these rings a new kind of colonies mixed with colonies of the old type, as is clearly to be seen in figure 5. The new colonies were whitish, shining and opaque and did not resemble either the S-type or the D-type. On replating, this type disappeared, giving rise to two new kinds of colonies, one forming rings with a whitish shining surface and surrounding a transparent bluish tiny central disc (fig. 12), another kind dull, cracky, opaque and in many other respects resembling the D-type. These were called SD (fig. 12). Subcultures of the new ring-forms in their turn produced SD-colonies. Thus the above-mentioned secondary types were apparently only transient except for the SD-type which turned out to be very constant. Repeated transfers in broth even at long intervals did not change the type nor was it possible to get the old forms back or to obtain new ones by keeping the plates for months. When, as will be noted later, the agglutination test also showed a resemblance between this new type and the old D-type, it was justifiable to suppose that the S-type had changed into a D-type. But an examination of the broth-culture in the hanging drop showed that the SD cells were motile. We may now, however, leave these plates for a moment.

As will be recalled, two series of broth-cultures were seeded on February 13 and samples removed and spread on agar-plates. The cultures on these plates did not show any aberrant types. The next time the broth-cultures were tested was on February 18. The S-plates were still unchanged, but every one of the D-plates showed a few shining sharp-bordered transparent colonies resembling the S-type. Sub-cultures in broth gave turbid growth. But in the hanging drop it was impossible to find one single motile microorganism. Thus the new type, being after all not a true S-type, was called DSh. At every new test the D-tubes showed such colonies in increasing number and the last time, on March 14, they were at least equal in number to the old colonies. The S-tubes on the contrary showed a great uniformity and the first variants were seen on March 5. These new colonies were in every respect identical with the above-mentioned type, SD, obtained from the secondary colonies of the old agar-plates of the S-type. Here we can now leave the S-type, since it did not show anything more of interest.

As for the D-type one of the plates showed on February 25 a phenomenon which was at first regarded as due to a contamination. Some of the dull colonies showed transparent fluid spots which in time spread over the whole colony and totally dissolved it. When the plate was tilted, the colonies of this type flowed to the lower level as a heavy fluid (fig. 11). The next test, on March 14 showed a few colonies of the same appearance in two other tubes and furthermore many of the dull colonies, at first of normal character, produced in two or three days on the border of the colony a fluid zone, as is to be seen in figure 9. At this time a new paper of Gratia on the Twort-d'Hérelle phenomenon appeared, in which he describes a mucoid type of *Bact. coli* obtained by treating the cultures with the lytic agent. His pictures and descriptions afford, I think, sufficient evidence that we are dealing with the same variant of *Bact. coli*; it appears, however, that in my case the mucoid quality is exceedingly pronounced. I venture to say this because, when transferred daily from tube to tube during a long period, the fluid character of the colonies gradually decreased and at last totally disappeared

in some tubes. Thus there is every degree of mucoid growth. It was interesting to see that on the disappearance of the mucoid growth the same dull colonies with a mucoid border-line appeared as in the beginning.

Evidently we are dealing here with a variant of *Bact. coli*. As it dissolved the old growth, a filtrate was tested but showed no influence on a culture of Type D. When kept for some days the mucoid growth dries up and can no longer be transferred to other cultures. Fishing from such plates gives a pure culture of dull colonies. On slides the mucoid substance is easily stained with gentian violet and even with methylene blue and then looks like mycelioid threads; in the hanging drop nothing but the bacteria is to be seen. As will be seen later this variant is very interesting because it is inagglutinable.

Finally one other variant may be mentioned, appearing in one of the D-tubes after the plating on March 14. On one plate were observed some tiny colonies, which, transferred into broth, showed slow growth, making the fluid turbid. Samples removed and spread on agar again gave a tiny growth of very irregular colonies, as is to be seen in figures 6 and 7. After a while, however, a luxuriant secondary growth appeared as demonstrated in the above mentioned figures. The difference between the tiny initial growth and the secondary luxuriant vegetation is, however, best visible in figure 3, which is a photograph of a slanted agar tube seeded from the original tiny colonies. Only two colonies have developed from the abundant bacteria that were seeded and for more than a week they gave only a scanty transparent vegetation, when at last a new fresh growth appeared in one of the colonies as a surrounding ring and in the other as a ring and a central heap. The behavior of the colonies reminds one strongly of the Twort-d'Herelle phenomenon. All attempts, however, to obtain an active filtrate failed.

Finally one may ask how long these variants are stable. In order to answer this question pure cultures of the variants DSh and SD were seeded in plain broth and samples tested on plates every eighth day. They are now more than two months old and both have acted in the main in the same way. Three

groups of colonies of different kinds are to be seen: (1) pure colonies of the variant itself; (2) pure colonies of the mother type, and (3) several colonies of new types not analyzed.

Plates kept for weeks produce, when replated, pure cultures. By transferring from tube to tube every second or third day it is also possible to keep the cultures pure in broth. Evidently it is easier to keep a type pure on agar-plates than in broth. That even on the plates a variation occurs has, however, been demonstrated above.

I have spoken above about pure cultures of the different types, although I am aware that the purity never can be absolutely sure. On the contrary it is very likely that the colonies of the D-types at least generally include some suppressed individuals of several variants. The impossibility of obtaining variants by fishing from the secondary droplets on the D-colonies indicates that this is probably the case. Nevertheless this must not be taken as an objection to the theory that a single cell is able to produce a growth of several variants.

By our experiments the D-type has been transferred to an S-type and conversely, except for the motility. The motility is, however, not a stable quality, as Gratia has shown. Thus there is no doubt that both the types of *Bact. coli* are only relatively fixed. This is extremely interesting, since, according to Gratia, the two types differ in virulence. In all our experiments the hydrogen ion concentration did not seem to be of any great importance for the variation, since, with one exception, the variants were the same in all tubes and occurred approximately at the same time.

AGGLUTINATION TESTS

Several agglutination tests were made, but it is not necessary to record more than one of them, since they do not differ in principle. The tested strains were one original pure D-type called D, one original pure S-type called S, one dull S-type called SD, one shining D-type called DSh, one mucoid type derived from a D-culture and called DF, and finally the above-described tiny strain called DT and its secondary luxuriously growing

strain called DTL. The bacteria were cultivated for eighteen hours on slanted agar and washed off by saline solution, mixed with the serum dilutions and kept in an incubator two hours before reading. Only negative or positive reactions at the different dilutions were recorded. A more elaborate method, such as Gardner's did not seem necessary for our limited purpose. The sera used were most kindly provided by Prof. A. Bergman, Director of the State Veterinary Bacteriological Laboratory. They were three in number, all polyvalent and obtained by injection into horses of coli and paracoli strains from calves. The results of the test are indicated in table 8.

TABLE 8

STRAIN	COLI SERUM 1					CONCENTRATION	COLI SERUM 2					CONCENTRATION	PARACOLI SERUM					CONCENTRATION
	1:50	1:100	1:200	1:400	1:800		1:50	1:100	1:200	1:400	1:800		1:50	1:100	1:200	1:400	1:800	
D.....	+	+	+	+	+	0	+	+	+	+	+	0	+	+	+	+	+	0
S.....	+	+	0	0	0	0	+	+	+	0	0	0	+	+	0	0	0	0
Sd.....	+	+	+	+	0	0	+	+	+	+	0	0	+	+	+	+	0	0
Dsh.....	+	+	+	+	0	0	+	+	+	+	0	0	0	0	0	0	0	0
Df.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dt.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dtl.....	+	+	+	0	0	0	+	+	0	0	0	0	+	+	0	0	0	0

The only trouble experienced in making the tests was a difficulty in getting a homogeneous suspension of the pure D-strain. Fortunately this strain showed an extremely strong and rapid agglutination which made the reading possible.

Table 8 shows very strikingly that "strains" obtained from the same single cell can differ widely serologically. This is not new but was shown before by Gardner and Walker (1921) and others with *Bact. typhosum*. Immunologic disparities of spores and vegetative stages of *B. subtilis* were shown by Mellon and Anderson (1919). As to the explanation of this phenomenon Theobald Smith and Reagh (1903-04) working with hog-cholera have advanced the hypothesis that there exist two kinds of agglutinin called "body agglutinin" and "flagella agglutinin," the first

produced both by motile and non-motile forms used as antigen, the second only by motile forms. The theory has not been accepted without reservation, since several authors have shown that different antigenic factors also exist in non-motile forms. Gardner and Walker, however, say that the hypothesis is not inconsistent with their experiments and I am convinced, too, that there is some connection between motility and agglutinability. My reasons are the following. The table seems to show that the more dry, dull and cracky the colonies are, the better is the agglutination and the more shiny, humid and mucoid the more refractive it is. When the type D becomes mucoid, it becomes at the same time totally inagglutinable. A similar phenomenon, perhaps in a less marked degree, is observed with the other strains. When the S-type produces dull colonies these are composed of bacteria more agglutinable than the mother "strain."

Evidently the surface tension of the bacteria is greatly changed when surrounded by a thick layer of mucoid substance. A phenomenon due to changes in the aggregation of suspended particles cannot of course be unaffected by differences in the surface-tension of the corpuscles.

The above mentioned observation that a thorough shaking with glass-beads of a broth-culture of type D produces a considerable spontaneous agglutination is of interest in this connection.

The power of the different sera mutually to impress the bacteria also changes with the variation of the microorganisms, as the behavior of the "strain" DSh shows. The paracoli serum does not possess any chemical complexes capable of producing visible aggregation in a suspension of the variant DSh. But the coli sera A and B possess this power. The fact that the mucoid "strain" in my experiment did not show any agglutination must not be taken as a proof that such strains are impossible to agglutinate, since Gratia, using sera prepared with the same bacteria that acted as antigen, obtained agglutination of mucoid cultures too. Nor are the microorganisms of the dull opaque colonies always easy to agglutinate, as the experiments of Baerthlein and

Gratia show. Serological tests specially directed toward this point would perhaps throw more light on the question. The conclusion of the argument must be, I think, that as motility and non-motility are connected with differences on the bacterial surface, it is probable that there must be some parallelism between motility and serological conditions.

Finally I want to call attention to an article by Jacobsen (1910) describing a strain of *Bact. typhosum* as being the cause of an epidemic in Denmark. The strain grows in very tiny colonies, on agar and is very slightly agglutinable, which caused difficulties in fighting the epidemic. Jacobsen thought that the delayed growth was due to chemical substance produced by repeated sterilizing of the agar, but Müller (1911) who examined the same strain, showed that this was not the case and that the tiny growth gave rise to secondary colonies of luxuriant vegetation, which were identical with the common type of *Bact. typhosum* and highly agglutinable. Evidently the behavior of my strains DF and DFL is a phenomenon of the same kind, corresponding in every way to that described by the authors mentioned.

Before finishing this article I wish to express an opinion with regard to epidemic diseases caused by microorganisms also living as saprophytes. In a paper on the prevention of pneumonia Rufus Cole (1918) writes: "So far as pneumonia due to these types of organisms (parasitic pneumococci) is concerned, the conditions do not differ essentially from those in diseases like diphtheria or cerebro-spinal fever in which the acquiring of the infectious agent is considered of primal importance." The observations with *Bact. coli* are not inconsistent with this point of view since they teach us that the variants once they occur retain their qualities with a relative heredity which is stronger the more rapidly the microorganism is permitted to multiply. The new discoveries about bacterial variation, however, show that the problem must be regarded also from other points of view and they illuminate at least one part of the mechanism which operates when saprophytic strains change into parasitic ones. Possibly studies on bacterial variation will in the future give a clue to the solution of the question as to how an epidemic dies

out. There are features in some epidemics, for example influenza, indicating that the parasitic qualities are hereditary only for a limited time. Perhaps a struggle for life among the variants plays a great rôle in the epidemiology. Investigations of Thomsen (1921) in cerebrospinal fever point in this direction. I myself have found in several cases of *Colitis ulcerosa* pure cultures of the motile form of *Bact. coli* only. Investigations are going on in order to find which type is prevalent in cases where *Bact. coli* acts as a parasite, for example in pyelonephritis.

SUMMARY

1. In an old laboratory culture of *Bact. coli*, two types of colonies were observed, one dull dry, cracky (D), and one shining, humid, transparent (S) the former being non-motile the latter motile.

2. The D-type showed growth in broth-tubes of a pH value varying between 5.0 and 8.6. The corresponding figures for the S-type were 4.3 to 9.4. The growing bacteria readjusted both the acid and alkaline tubes to a slightly acid reaction. A too high acidity, however, killed the growing bacteria before an adjustment of the hydrogen ion concentration had occurred. The bacteria in the tubes of a hydrogen ion concentration too high or too low to permit growth were rapidly killed.

3. The high and low hydrogen ion concentration produced an increased bacterial lag and a diminution of the total amount of growth despite the readjustment of the reaction.

4. In broth-cultures of varying hydrogen ion concentration permitted to age, the D-type produced variants rapidly, the S-type slowly. Some of the D-variants resembled in every respect except motility the S-type and conversely the S-variants resembled the D-type. On agar-plates the variations were visible as secondary colonies.

5. The variants could be kept pure for months by repeated transfers from broth to broth or on agar plates. When left in broth-tubes they produced colonies of the mother type and new variants.

6. The D-type was agglutinated at high, the S-type at a very low dilution by polyvalent coli serum. The shining variants of

the D-type were less agglutinable than the mother type and the full variants of the S-type conversely more agglutinable. One extremely mucoid variant of the D-type had totally lost its agglutinability. This phenomenon seems to indicate that the variation in agglutinability is due to changes in the mucoid layer surrounding the bacteria. A broth-culture thoroughly shaken with glass-beads showed spontaneous agglutination.

7. One variant of the D-type showing a very tiny and barely visible growth and a bad agglutination produced a luxuriant secondary vegetation of another type agglutinable at higher titer. This phenomenon corresponds to the observations of Jacobsen and Müller with *Bact. typhosum*. The secondary vegetation sometimes formed beautiful rings round the tiny almost invisible original central growth.

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PLATE 1

FIG. 1. A one-month-old agar plate of the S-type showing a few secondary colonies forming rings.

FIG. 2. An agar plate of the D-type one month old demonstrating secondary colonies in the form of droplets.

FIG. 3. A three-weeks-old slanted agar culture of the type DT containing two colonies with an initial tiny growth and a secondary luxuriant vegetation, forming in one of the colonies a surrounding ring, in the other a ring and a central heap.

FIG. 4. An agar plate two weeks old seeded with a sample removed from the 7th tube in the series of the D-type on March 5th and containing not only several big colonies of the dull type with secondary droplets but also small colonies of a variant resembling the S-type. Fishing is made from both types and the needle has cracked the dull, dry, big, colony. From the small ones, on the contrary humid masses have been dragged out, only owing to the paste-like consistency of these colonies.

FIG. 5. Two kinds of colonies obtained by fishing from the secondary colonies on the agar plate indicated in fig. 1. The dark ones are the variants, the others resembling the mother type in every respect.

FIG. 6 AND 7. Colonies on three-weeks-old agar plates at different magnification of the type DT. The tiny transparent growth is seen in the periphery of the irregularly denoted colonies, the central parts being occupied by a luxuriant secondary vegetation.

FIG. 8. A ten-days-old agar plate of the DF-type. The mucoid, fluid parts of the vegetation are mostly dried up. The non-mucoid growth shows secondary colonies of the same sort as in fig. 2.

PLATE 2

FIG. 9. Mucoid growth on the border of colonies of the dull type. The plate is two weeks old.

FIG. 10. Two broth-cultures of the series seeded on February 13 being two months old. The D-type to the left, the S-type to the right.

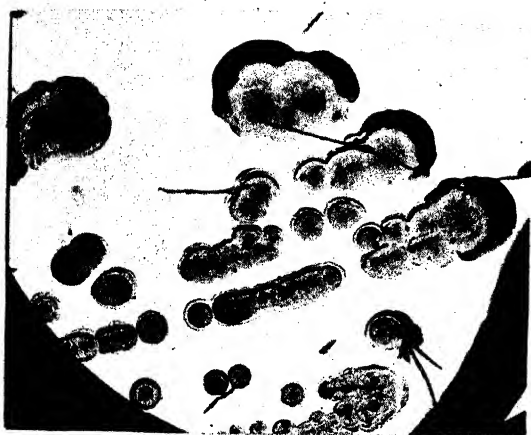
FIG. 11. Mucoid growth dissolving the dry colonies.

FIG. 12. Colonies obtained by fishing from the dark colonies demonstrated on figure 5. The ring forms are a transient variant, the others a very stable one of a type resembling the D-type and signed SD.

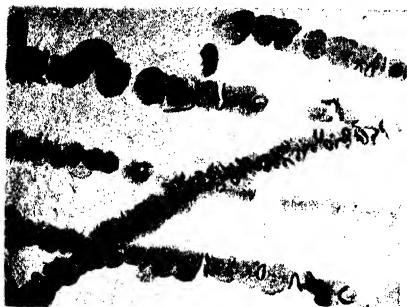
FIG. 13. Another part of the plate explained in figure 8.



(Bergstrand: Variations of *Bacterium coli*)



9



11



12



10



13

WHAT CONSTITUTES EFFICIENCY IN RESEARCH?¹

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In selecting this subject I realized that it was not a new one, and that nearly everything that could be said on the topic had already been said in one way or another. If I had known then as well as I know now how very much had been said, how many presidential addresses on the organization of science fill the pages of *Science*, I think that I would have had compassion and spared my audience this last straw. And yet, in going through these numerous addresses and letters, I cannot help thinking that in some cases the writers have failed to distinguish between the real organization and the defects of the organization, between the meaning of the word "efficiency" as used in business, and what constitutes efficiency in research. I use the words "organization" and "efficiency" deliberately, realizing fully the extent to which they have been overworked and the antipathy which they arouse in the minds of some efficient directors of well organized laboratories.

One does not need to read many pages of the journals in which scientists exchange ideas to realize how many look upon an organization as applied to research as something in part, if not wholly, evil. For them the word "organization" is associated with supervision, with administrative duties, with everything that is the antithesis of the freedom of thought and action assumed to be essential to real research.

We are frequently reminded that many of the great men of science have worked alone and the inference is drawn that their accomplishment was great because they were unhampered by

¹ Address of the President at the Twenty-fourth annual meeting of the Society of American Bacteriologists, Detroit, Michigan, December, 1922.

an organization. Someone has painted an amusing picture of Newton ordered by the director of a modern organization to make a progress report on his theory of gravitation. There seems to be a fear in some quarters that if one is truly scientific he cannot be "efficient." This aversion to efficiency has no doubt been accentuated by the association of the word in the past few years with a systematized effort to increase the output of machines and manual labor. From this we have come to associate the word "efficiency" with stopwatch studies, with close supervision, with time cards, and with detailed daily reports. Organization and efficiency remind us of the proposal, seriously made during the war by a prominent American, for a research institution surrounded by a high brick wall and manned by a force of scientists working in eight-hour shifts. We are afraid that in an effort to increase our efficiency, our freedom of working out our problems in our own way, and at our own convenience will be curtailed.

It must be admitted that some of the fears incited by the word organization have a basis in fact. It is true that many of our ablest investigators have ceased to be producers through the encroachment of administrative duties incident to a growing organization. The preëminent scientists of the past—the Newtons, Darwins and Pasteurs were not forced to pay this penalty for proved ability, and continued to be producing investigators throughout their lives. We can estimate now what the world would probably have lost if these men had been obliged to devote the greater part of their energies to administrative work. If Pasteur had been the director of a modern laboratory, preparing reports and estimates, attending meetings of committees and research councils, speaking at dinners and women's clubs, would his name have become so interwoven with our daily life that its use may be taken as a measure of the state of civilization of a people? But little can be proved one way or the other by what did happen, or what might have happened to the Newtons and Pasteurs; they were extraordinary men and would have risen above the distracting influences of an organization just as they rose above the lack of material equipment.

Certain evils have become so associated in our minds with a large organization that we are inclined to look on these evils as inherent and unavoidable. Red tape is not confined exclusively to the business of the government, but may be found entangling the work and impeding progress in any large organization. It is safe to say that the greatest difficulties which the average investigator has to overcome are not involved in his problem itself, but are those thrown in his way by man-made organizations. Usually these obstacles are constructed in the name of efficiency and by those who are employed to assist, not to obstruct.

I could cite from my own experience many instances which illustrate how a highly complex organization frequently defeats the end for which it was created and these, no doubt, could be matched by any one connected with any large institution. In the course of an investigation to which a vacuum pump was essential the shaft of the pump was broken. Now, an inefficient scientist, unhampered by a highly organized supply system, would have sent a messenger to the nearest hardware shop for 2 feet of 1-inch steel and in a few hours the machinist could have made a new shaft; but a purchasing committee which, by its own confession, is highly efficient, had protected the financial interests of the government by contracting with a dealer in Pittsburgh, for a year's supply of this particular kind and size of steel. Consequently, it became necessary to make a formal request for a requisition on this dealer, a process which in the normal course of events requires several days, and then to wait the pleasure of the dealer in filling this large order. Fortunately, in this case, the purchasing committee had not foreseen the possibility of needing parts for this particular kind of pump and we had the privilege of ordering a new shaft from the manufacturer. By using the telegraph it was possible to reduce the delay to a few days.

Many of the complications of organization have resulted from the application of the popular fallacy that efficiency and economy can be promoted by the consolidation of small units into larger ones. One of the inherent evils of a large organization is the tendency of the clerical element to dominate the scientific force. As the organization grows the worker becomes further removed

from the source of authority, the paths through which the business of the organization must pass become devious and full of obstacles, and the hands of the worker become tied by regulations and precedents. The economy of consolidation is also more frequently apparent than real. If the function of a library is merely to distribute books at the least possible expense, no doubt this end could be best accomplished in a centralized library, but it is better to appear extravagant by spending money for branch libraries than to have a laboratory force work for weeks or months on a problem because, through difficulty in getting books and periodicals, they are ignorant of the fact that the problem has been solved.

One of the windmills against which the organizers most frequently tilt is the duplication of work. It is easy to see that in certain types of work duplication does become a great evil, but in research work it is not duplication but the lack of duplication that is to be avoided. Few investigations lead to wholly correct conclusions and new results become established as facts only when they are confirmed by several workers. It is only by the cumulative effect of disconnected attacks on a problem from different angles that progress is made. This may be true even in a single laboratory in so far as it involves application of different types of mind to the same problem.

Evidently the evil of which the writers of the presidential addresses stand in greatest fear is a curtailment of the investigator's inherent right to develop his own ideas in his way.

Dr. A. Franklin Shull (1), speaking of ways of discouraging or limiting freedom of research says that "one of these ways is the appointment of an investigator to a position for the purpose of studying a certain problem." He cites a terrible example where this actually occurred and adds, "By the terms of his appointment his energy could be directed into other channels only with the permission of his superior officer. Such direction from above could be justified only in the case of an assistant or an investigator on temporary appointment, not in the case of a permanent colleague."

I must confess to many transgressions along exactly this line, and for my peace of mind would like to have someone tell me how the very definite problems which confront us can be solved without thus limiting freedom of research. The money with which the investigator's salary is paid, and his equipment purchased, may have been provided for the study of a very definite question and it does not seem unreasonable to expect him to stick to his last. The particular method by which he attempts to solve the problem is another question, but even here many beginners are unfortunate in having had too much freedom. Some men are successful only because, throughout their career, a skilful pilot has held them to the course.

William Morton Wheeler (2) after a very entertaining description of the characteristics and habits of the genus scientific investigator, concludes that "Attempts at organizing investigators must fail because their highly specialized activities depend to such a great extent on their peculiar native aptitudes or capacities."

While I must admit some evidence of temperament, my experience has led me to believe that investigators do not differ in their essential characteristics from other people.

It is true that the qualities which make a successful investigator are different from those which make a successful salesman, or financier, or mechanic, but are we ready to admit that they preclude the possibilities of systemization or coöperation?

However, the trend of the presidential addresses is not entirely toward individualism as opposed to organization. Milliken (3) says, "The extraordinary rapidity with which scientific developments were made during the war was unquestionably due then, first, to the forming of these highly competent research groups; and, second, to the establishment of effective channels for the coöperation between these groups."

This process of organization and coöperation, while greatly stimulated by war conditions, is not an especially new movement.

Less than a quarter of a century has seen the development of great institutions with resources, equipment and organization undreamed of by our fathers. We have voluntarily formed what

has been described as a "superorganization of superorganizers," the National Research Council. We are told that we must coöperate. The efficiency of existing organizations is to be increased by coördination and consolidation. The problem of organization is continually with us whether we welcome it or not. No laboratory is so small as to avoid it. When an investigator acquires an assistant it comes to him. When a man becomes an assistant he faces an organization problem and his is, perhaps, the more difficult one because he lacks the authority to put his ideas into effect.

In building up our organization there are certain obvious ends to be gained and certain obvious evils to be avoided. We perfect our organization to obtain efficiency. Our funds are usually provided for definite purposes. We have a program of work planned, a particular end to attain, and the money available should be so expended that it will carry us the greatest possible distance on the road to that end. Of even greater importance is the necessity of expending the energy and ability of the workers so that they will yield the greatest returns in the advancement of human knowledge.

The danger in any organization of scientific work lies in the tendency to submerge the individuality of the worker. In such organization we are not dealing with machines, nor with piece workers. In research work the unit of the organization is a highly developed human mind. The product which our organization turns out is the result of the thought of an individual worker, and just so far as the organization inhibits or distracts these minds from their true course it is inefficient. On the other hand, the organization promotes efficiency so far as it tends to permit and to stimulate originality and freedom of thought in the individual worker and at the same time to coördinate and concentrate the activities of the several investigators on the particular problem in hand.

In considering means of attaining this end we must keep in mind the distinction between the methods of the factory efficiency engineer and those which must prevail in the laboratory. In the factory the road to efficiency leads through system, routine,

supervision, coördination of men and machines, office records and elimination of unnecessary motions. In the laboratory efficiency is obtained by reducing supervision to the lowest point compatible with the ability of the investigator; by removing him from the distractions of report writing and routine office work; by surrounding him with an atmosphere conducive to study and meditation; by providing him with the special equipment necessary to the solution of his problem and by promoting a spirit of coöperation which will unite the individual investigators into a compact body working together on a single group of problems. We should always keep in mind that the staff is the laboratory not the building or the equipment which are frequently exhibited as examples of a great laboratory. Liebig is said to have done some of his best work in a kitchen; Pasteur, working in a dark basement was directing a great laboratory.

However, in building our organization on the individual worker we must keep in mind that there are diverse kinds and conditions of men. Scientific investigators may be divided into two classes, the leaders and the followers. By "followers" I intend no disparagement of the great class of scientists to which most of us belong. Indeed, it would be very difficult to decide which is of the greatest value. Daniel Boone and his fellow explorers who blazed the way through the mountains and the pathless forests into the Kentucky valleys made a great state possible but without the settlers who followed in their footsteps and turned the wilderness into productive farms and thriving cities their efforts would have been valueless and forgotten.

The leaders are the exceptional men, with imagination, who have the faculty of grasping the significance of phenomena that to the ordinary worker seem trivial or incomprehensible, and who, by a series of brilliantly planned experiments, push back a little further the wall of darkness that marks the limit of human knowledge. For a man of this type the organization must afford wide latitude in the selection of problems and in the method of solving them. On the other hand, there are many men who, if left to the dictates of their own imagination, flit from one unfinished project to another and accomplish little beyond their

own entertainment, while they may, under the right leadership, become good investigators. For these men a more rigid project system is necessary. For the great majority of investigators who depend for their progress on industry, perseverance and logical thinking all that is necessary is the essential equipment and an opportunity to develop their particular problem.

In developing an organization consideration must also be given to the type of work to be done, although the underlying principles apply to all cases. The only real difference in research institutions is in the primary object to be gained. Is the institution designed to produce investigators or the results of investigation? Each will train men and each will increase knowledge, but the organization and methods of work will necessarily be along different lines. The classification into laboratories for pure science as contrasted with applied or industrial laboratories is not basic but merely indicates the scope of the laboratory. In the laboratory of pure science knowledge is sought for knowledge's sake. The laboratory is in a way incomplete, a fragment of a laboratory. The investigator, through inclination, lack of facilities or appreciation of the possible application of his results fails to carry his investigation to the point at which the value is evident.

In the words of Huxley "What people call applied science is nothing but the application of pure science to particular classes of problems." The applied laboratory which does not develop its own problems in pure science must begin where others have left off and carry the investigation on to its application. The more highly organized of the industrial laboratories have provided for what we will continue to call, for lack of a better term, pure science, wisely permitting great latitude in the selection and development of problems. The wisdom of this course is already evident in the fundamental results of great scientific and economic value which have come from some of the investigations of these laboratories. No one can foretell the results of investigations of this nature or estimate their value, not even their pecuniary value to the company which profits by the investigation. The history of the progress of science in the past teaches us that the great

discoveries of the future, will be along lines which cannot be conceived in advance. Discoveries, which to the ordinary layman are the creatures of a single master mind, are the natural sequences of a long series of investigations. They are not isolated points, but the apexes of pyramids shining in the sun of publicity. Few, if any, of these investigations were undertaken with even a hope that their application would benefit humanity. The invaluable x-ray was only an incidental result in a series of investigations on the nature of the electric discharge. These studies were made possible by the perfection of methods of obtaining high vacua and this in turn was developed to permit the determination of the atomic weight of thallium. The public knows of the "discovery" of x-rays, but is not interested in the nature of the electric discharge and knows, and cares, nothing about the atomic weight of thallium.

The research laboratories of the universities are usually organized on a somewhat different basis from the industrial laboratory in that the main function is teaching and the results of research are incidental. The limitation of many of the investigators both in time and experience, and the necessity of selecting problems which will give training in research to successive generations of students, narrow the scope and determine very largely the nature of the organization.

In an industrial laboratory, while there is frequently necessity for training new men, the organization is based entirely on research for the sake of the results.

A complete research organization has four functions: (a) The study and development of new laws and fundamental truths without regard to their application or usefulness; (b) the development of possible uses for new knowledge, and the solution of problems which arise in the varied activities of mankind; (c) the trial of results obtained in the laboratory under conditions which will test their applicability to practice; (d) the dissemination of the results obtained. Few organizations are complete in this sense. Some confine themselves to the study of fundamental science only; some build on the basic facts developed by others; few have the facilities for applying what they learn under working conditions.

It is very easy to make diagrams on paper showing how, in a logical way, this should be done, but diagrams, no matter how well done, cannot really represent human beings, and as Mr. Little has observed, "There is danger in an organization chart—danger that it may be mistaken for the organization." Whatever type of organization is chosen, the executive functions will center about the administrative head of the institution. We are in the habit of designating this official as a director, although the term does not fit harmoniously with our conception of scientific research. The motive power for research must come from within and it can be directed only in a very limited way. The head of a research organization must be, not a director but a leader. His function is not so much to direct research as to recruit a staff; to provide the necessary material equipment; to maintain an atmosphere conducive to constant application, and to coördinate the work of the investigators on the problems of the laboratory. The members of the staff are his associates, not his assistants, and he serves the ends of science best when he stands between them and the distractions of correspondence, finances, and report writing.

The form of organization which has been most used in the past follows the lines of the basic sciences. This requires departments of chemistry, bacteriology, pathology, or whatever sciences may be involved. It necessitates arrangement for coöperation between departments on any problem involving more than one science, something which is very easy to show in a diagram, but very difficult to obtain in practice. This system has the advantage of making it possible to have an especially strong man responsible for the work in each branch of science, but carried to its logical end establishes artificial barriers which impede progress.

In the early days of the Department of Agriculture there was a Division of Microscopy where, presumably, all of the microscopic work was to be done. The fallacy of this arrangement was, apparently, early recognized, but there grew up later the Bureau of Chemistry, which was to do all of the chemical work for the Department. The difficulties of an organization which confined all of the work in a basic science to one laboratory were not so

evident but when different bureaus, in order to make progress on their problems, found it necessary to establish chemical laboratories this arrangement was abandoned in its turn. Such an arrangement is illogical in a small laboratory, and becomes more and more illogical as the organization grows larger. In solving a biological problem chemistry must be looked on only as a means to the end, a yard stick by which the investigator measures and explains the changes which he observes. The chemical work should be under his control as completely as any of the other tools essential to the solution of his problem.

The organization which usually evolves, if a laboratory grows up naturally, is a force of workers with various qualifications grouped around each problem. A chart showing an organization on this plan usually develops into a fine collection of wheels within wheels. In practice, however, it does not work out as simply as the diagram would indicate. The nature of the plan requires a continual shifting and readjustment of men as the problem develops, or is completed, or abandoned, and new problems are taken up.

It is not always feasible to assign an old and more experienced investigator to work under a younger man who may be responsible for a problem. Some excellent investigators do not have the knack of managing assistants; others do not work well independently, but require close supervision.

The organization around the problem becomes then a question of fitting the arrangement to the workers available, deciding each case on the basis of its own peculiar conditions. It is entirely possible to obtain in one laboratory harmonious and efficient work with the various problems organized in different ways; in one with workers of more or less independence of action responsible to a leader who is in turn responsible to the director; in others with independent workers coöperating and each responsible to the director for his part.

A minor but essential part of the laboratory is that which deals with what may be called the accessory branches, through which the material needs of the organization are provided. These are not coördinate with the investigational staff, but are service units existing only to expedite the work of the investigators.

A general in planning a campaign provides means of transportation, and lines of communication to keep his troops supplied with ammunition. This branch of the service is secondary, but of the most vital importance. If it fails the campaign fails. Its function is to get ammunition to the fighting troops in sufficient quantities and at the time it is needed. The color of the wagons in which it is brought is of little importance. It is of little comfort after the battle is lost for want of shells to know that the records of the ordinance division are complete and accurate.

The clerical force of a research organization performs a similar function. Its function is not to establish channels through which the business of the laboratory must go regardless of consequences, but to provide ways of getting the ammunition to the front when it is needed. The office that has allowed a blind adherence to records and regulation to obscure this end has outlived its usefulness.

The method of administering this service is a difficult one to arrange satisfactorily. Some of the large research institutions relieve the director of all responsibility for financial matters, making the accounting and purchasing division responsible directly to the governing board. Some go to the other extreme, and not only make the clerical force responsible to the director of the laboratory, but place it under the immediate direction of a man of scientific training. The former method would seem to offer large opportunity for the exaggerated idea of the importance and authority which is the great evil of the service branches—the latter should enable the director to make the clerical force what it should be, a servant to the investigational work.

What has been said of the clerical force applies also to the library service, except that with few exceptions the librarians appreciate their relation to the investigational staff and any failure to function properly is usually due to faulty organization rather than to lack of inclination to be of assistance. The contact between the staff and the library must necessarily be a close one. Nothing is more essential to efficiency in research than ready access to the literature. Library administration is not clerical work, but requires special training, a knowledge of

languages, of methods of classification, and above all a wide acquaintance with scientific literature. The library should not be controlled in any way by the clerical force, nor should it be responsible to any particular branch of the laboratory. Its contact with the force should be through the director. It should be so organized that the periodicals are available promptly and the library staff should be in a position to be of real assistance in searching the literature.

A third auxiliary branch which should not be overlooked is the mechanical shop. Even in these days there are scientists whose time is more valuable than that of mechanics and it is not economy to allow work to wait for special apparatus or repairs. In a small laboratory the shop may be only a well equipped work bench; in some lines of investigation the shop is really part of the laboratory, and should be complete and adequately manned. A day rarely passes in a laboratory that some small construction or repair job does not arise. The mechanical branch should be available to take care of this work without unreasonable delay.

Although the results of research are of no immediate value unless an application can be shown, few laboratories outside of the industrial institutions have any organization for the specific purpose of applying new facts and theories to practice. It does not necessarily follow that results are lost because no special provision is made for their utilization, but it is no doubt true that many valuable results are overlooked, and forgotten, because the investigation could not be carried to its logical conclusion.

If Mendel, instead of being an example of the highly individualistic type of investigator, had been a part of a modern research organization, the basic law which he discovered would not have been buried for years, but would have been put into practice at once to the great advancement of plant and animal breeding. Some investigators do not continue their investigations when they reach the applied stage because their tastes lead them to new paths in unexplored fields. In other cases the necessary equipment may not be available, or the branch of human activity to which the results may apply not at hand. The equipment needed may be a factory, a farm, a water system, or a hospital. The

expense and in some investigations the danger to human life may be so great that it becomes advisable to follow the laboratory study with larger but still limited and controlled experiments.

This is generally recognized in industrial laboratories where provision is usually made for carrying an investigation through the laboratory, through a miniature factory test and finally through a full scale factory operation.

In bacteriological work the same course is frequently followed, although the analogy may not be evident at first glance. The soil bacteriologist has his pots and experimental plots, the pathologist his animal rooms and hospital wards.

The efficiency of many other lines of investigation would be greatly increased if definite provisions could be made for carrying investigations to real completion without unnecessary delay. While in some cases it is real economy to encourage an investigator to turn to new problems of a basic nature, leaving the application of his results to others, as a rule the man who develops new principles is the one best fitted by interest and knowledge of the subject to apply them.

Many processes which work well in the laboratory develop defects when tried on a large scale. Many new methods of real value have never gotten beyond this stage, because there was no one with sufficient interest or technical knowledge to adapt the process to the new conditions. Thus there is a great economic loss which can be overcome by proper organization.

And, finally, to complete the work it is necessary to carry the results to that part of the public which is directly concerned. Is the task completed when a technical paper is published in a scientific journal? If its value lies in its use in investigational work it is usually sufficient to make it available to other scientists but if it is something which affects established practices it is only a beginning. The inertia of public opinion cannot be overcome so easily. Even the medical profession, accustomed as it is to rapid advance, cannot always be reached by the printed word alone. New developments in agricultural science permeate very slowly through the journals, the text books, and the teachers to the farms.

The necessity for actual demonstration has become more fully realized, particularly since progress in all fields of science has become so rapid that practice lags behind. The medical profession was convinced of the value of typhoid vaccination by large scale demonstration in the army. The efficiency of administration in introducing new methods in agriculture is now generally recognized and practiced. There is, however, too often a lack of continuity between the investigation and the demonstration. The investigator lacks the facilities for demonstrating his new methods, and the demonstrator is not always sufficiently familiar with the investigational work to select the most valuable results or to avoid mistakes and failures. Real efficiency is obtained when an investigation can go direct from the laboratory to the demonstration stage, with sufficient control retained by the laboratory to insure the use of correct methods.

In the laboratory with which I am connected, if you will pardon the personal allusion, an organization has been perfected which makes this possible. I may cite as an illustration the case of a bacteriological study which proved the relation of certain types of bacteria to the production of the characteristic flavor in Swiss cheese. Through coöperation with a technical man the value of these bacteria in cheese making was determined by miniature factory tests in the laboratory. While this demonstration was very successful, if the work had been stopped at this stage it would still be in the "of scientific interest, but doubtful applicability" class. Everyone who has had experience with pure cultures in the industries knows that there is a wide gulf between the propagation of cultures by a bacteriologist in a laboratory, and by a hand in a factory. Preliminary trials in factories proved that the use of these cheese cultures was no exception. Accordingly, some years were given to operations on a commercial scale in a factory controlled by the laboratory. This not only served to develop and correct some of the difficulties which occur under factory conditions, but also demonstrated the commercial value of the cultures and incidentally trained men for field work.

The actual introduction of these methods into the industry was passed on to another section of the organization, with sufficient control of the methods retained by the laboratory to insure their correct application. The method of introduction consists in demonstration in the factory of the value of the cultures and instruction to the operators in their use.

Thus, we have an example of an organization which provides for basic investigation, for laboratory scale manufacturing tests, for trials under controlled commercial conditions, and finally for carrying the results to the industry by demonstrations and instruction.

If I should attempt to summarize these rambling observations in one sentence I would say that in promoting efficiency in research the essential thing is not the form of the organization but rather the spirit in which it is applied. The organization should be like a roadway over which the investigation passes, not a structure in which it is confined.

Wheeler, from whose address I have already quoted says: "And as the surface of the planet becomes more and more densely covered with its human population, it becomes increasingly necessary to retain portions of it in a wild state, i.e., free from the organizing mania of man, as national and city parks or reservations to which we can escape during our holidays from the administrators, organizers and efficiency experts and everything they stand for, and return to a nature that really understands the business of organization."

Dr. Wheeler has used a more apt illustration perhaps than he realized. The parks to which he would escape to allow his primordial instincts free play are possible only through an efficient organization. The forest through which he roams unhindered has been saved from commercial exploitation by this organization; a fire warden in some isolated lookout station is watching for the first sign of the conflagration which his primitive instinct may have started, and a carefully organized and equipped fire-fighting force is waiting for the telephone message saying that its services are needed at a certain spot; this organization stocks the streams with fish, protects the game, and constructs

roads and trails over which he can flee from the dreaded organizer; in short, it makes it possible for him to escape, as he expresses it, "from the organized routine of our existence."

This conception of a park fits so well with my idea of a properly organized laboratory that I can say with Gratiano, "I thank thee for teaching me that word."

In the ideal research organization the investigator can follow the lure of his elusive problem protected from the distractions of business and finance and propaganda: he can make better progress and enjoy it none the less because the paths have been made smooth and the camp grounds supplied with wood and water. A sense of security should come from the knowledge that a well organized system is protecting him from the consuming conflagration. He may even find it pleasant to exchange notes with the watcher when his path leads him to the look-out station which connects him with the outer world.

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STUDIES ON SALT ACTION

VIII. THE INFLUENCE OF CALCIUM AND SODIUM SALTS AT VARIOUS HYDROGEN ION CONCENTRATIONS UPON THE VIABILITY OF *BACTERIUM COLI*

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OBJECTIVES

In an earlier paper (Falk, 1923) our present state of knowledge in regard to the physiological influence of mineral salts upon bacteria has been somewhat comprehensively reviewed. The experiments here reported were designed to determine with some exactness the effect upon bacterial viability of sodium and calcium chlorides, alone and in combination. Our work has extended over a long period, the first preliminary communications (Winslow and Falk, 1918) having been made over four years ago. The early experiments at times yielded conflicting results and it was only by a careful study of the hydrogen ion factor (Falk, 1920) that we succeeded in explaining these inconsistencies along the lines since so well worked out for problems of bacterial growth by Holm and Sherman (1921) and Sherman and Holm (1922). The study presented here deals with the viability of *Bacterium coli* in distilled water, in various concentrations of NaCl, and in various concentrations of CaCl₂ with varying hydrogen ion concentrations for each solution; while a following paper (IX) deals with viability in various mixtures of the two salts mentioned above.

• TECHNIQUE

All of the experiments here reported were conducted with a single strain of *Bact. coli* which was isolated from a polluted stream near New Haven in the autumn of 1916 and has been

used ever since for various studies of viability conducted in this laboratory.

The bacteria needed for each experiment were cultivated on standard nutrient agar slants, or Petri plates or in Kolle flasks at 37°C. for sixteen to twenty-four hours. The growth from the surface was washed off in water or in the appropriate salt solution, shaken for three to five minutes to break up clumps, filtered through paper or absorbent cotton and then added in 1 cc. portions to the bottles of sterilized water or salt solution in which the viability was to be tested, the solutions having previously been warmed to a temperature of 37°. Quite uniformly the concentration of bacteria at the beginning of each test was 20,000,000 to 40,000,000 per cubic centimeter. Only occasionally was it somewhat lower or higher. Plates were made on agar one minute after seeding to determine the initial number present. The bottles were then replaced in the incubator at 37° and removed for the estimation of the number of surviving organisms at various desired intervals. All counts were made on a standard nutrient agar after incubation for twenty-four hours at 37°C.

The water used in these experiments was carefully distilled from a Barnstead still, or was twice redistilled in glass vessels and again redistilled from glass and into a block tin condenser. Whenever tested it gave a negative test for ammonia with Nessler's reagent. The hydrogen ion concentration of the water used in the earlier experiments was generally between pH 6.4 and 6.8 but was not recorded in regular routine. In certain other tests the initial acidity of the water was higher, usually between pH 5.2 and 5.8.

On the addition of the bacterial cells this reaction would be shifted to a pH of 8.0 or above by the alkaline dissociation products of the suspension. Later there was a tendency for the reaction to return to a zone of hydrogen ion concentration between pH 7.0 and pH 7.4, after an incubation of from one to forty-eight hours. The effect of initial reaction is however often an important one; and in our later work this factor has been carefully controlled.

In those experiments in which we recorded the pH of the solutions, the measurements of acidity were made by the usual

colorimetric procedure, either before or after the addition of the suspension of bacteria according as the pH measurement value "before" or "after" seeding was desired. When the pH of a solution was readjusted, great pains were taken to introduce as few new ions and in as small a concentration as reasonably rapid work permitted. In adjusting the pH of bacterial suspensions in water and in sodium chloride solutions, HCl and NaOH were used. With calcium chloride solutions HCl and Ca(OH)_2 were used. The additions of acid or alkali were made by measuring the pH of an aliquot from the test solution, consulting a titration curve of a similar prepared solution to determine the quantity of acid or alkali to be added to give the desired pH and reading the pH of another aliquot after the appropriate addition. Excepting for the water suspensions, the addition of these reagents involved the introduction of no new ions. Because of the comparatively low buffer content of the suspensions in water or in dilute aqueous solutions of these salts, the amounts of acid or alkali added to give the stated pH values were exceedingly minute and the increase in concentration of Na^+ and Ca^{++} ions in the salt solutions incidental to these additions was generally entirely negligible by comparison with the concentration of these ions already present.

Following the work of Cohen (1922) we made some studies of viability in dilute (M/500) buffer solutions. This particular group of studies was carried only far enough to indicate that the buffers, even in such dilution, were producing effects specific to themselves. The results of these experiments are therefore omitted from this paper on the viability of *Bact. coli* in pure water or in solutions of single salts. The study of pH influences upon viability in the absence of buffers is much more difficult and the results from individual experiments are more uncertain than in their presence, but it makes possible observations of certain phenomena which it would be otherwise impossible to observe.

The sodium chloride used in our earlier investigations was purified by treatment with calcium hydroxide (to remove magnesium) and with sodium carbonate (to remove calcium and barium), and was then recrystallized from pure concentrated

hydrochloric acid and from pure water; the calcium chloride was twice recrystallized and the crystals dried in a current of air and in the drying oven. In our later experiments we found that "chemically pure" (analyzed) commercial preparations of these salts were entirely satisfactory when the limiting concentrations of impurities were low.

VIABILITY IN DISTILLED WATER

First of all it was important to determine the behavior of the particular strain of *Bact. coli* used in our experiments when suspended in distilled water free from the presence of notable amounts of mineral salts. An earlier investigation of the viability of this strain by Winslow and Cohen (1918) had shown that it suffered only a slight reduction in numbers when suspended in water for twenty-four hours (82 per cent surviving) but that after this period it gradually died off, only 49 per cent remaining after three days and only 5 per cent after five days.

We ourselves conducted 37 different experiments of this kind during the first years of this study, the results of which are presented in table 1. The pH values at the head of each column indicate the acidity to which the solutions were adjusted immediately after seeding the bottles of water with the bacterial suspensions and at which they were maintained by readjustment of reaction at each observation time if they showed a variation of 0.2 or more from the stated pH. A uniform tendency for the pH to shift towards the zone 7.0 to 7.4 is found here as well as in the experiments with suspensions of *Bact. coli* in sodium and calcium chloride solutions. This point will be treated more fully later. Tests under the column headed "x" were early experiments conducted in solutions, initially alkaline, but in which the pH was neither recorded nor readjusted.

The figures given for the per cent of organisms surviving at each period represent in each case the average of from 2 to 11 tests conducted under the conditions specified, the first seven columns including tests made at known pH values, the last column the earlier tests in which hydrogen-ion concentrations were not recorded. In this and succeeding tables a heavy rule

has been drawn to indicate the range of time and hydrogen-ion concentration most nearly corresponding to a one third destruction of bacteria. There are of course occasional irregularities in the viability curve which make the establishment of this one-third reduction point somewhat conjectural in certain instances as in the columns of table 1 for pH values of 5.0 and 6.0.

It appears from table 1 that at pH 6.0 the reduction in the number of bacteria is very slight, while at pH 5.0 it is somewhat greater; in more acid or more alkaline solutions the viability decreases rapidly. This table, as indicated in its lowest line,

TABLE 1
Viability of Bact. coli in distilled water

HOURS	PERCENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	x
1	87	88	84	92	68	77	79	196
3	39	71	74	66	54	24	52	134
6	4	48	64	30	24	8	12	78
9	1	68	82	7	17	5	12	89
24	0	6	77	2	23	3	10	57
Number of experiments...	2	2	4	2	10	2	4	11

includes the averages of 37 experiments; for the 9-hour period we conducted a more extensive series of tests, this period being chosen because our earlier work had indicated that the physiological influences which we were desirous of measuring were most clearly evident at that time. In table 2 we have collected all of our nine hour data, including those cited in table 1, to give a final table which represents the results of seventy-nine tests.

The curve which expresses the relation between pH and the viability of *Bact. coli* in distilled water is obviously smooth and indicates the existence of a zone of physiological tolerance be-

tween 5.0 and 7.0 which is exceeded more abruptly at the acidic than at the alkaline extreme.

There is some discrepancy between the results of the first 37 tests (table 1) and those of the whole 79 tests (table 2), as would naturally be expected in dealing with a problem of such complexity as the viability of bacteria. Only at pH 7.0 however is this discrepancy serious, the first group of tests showing a 17 per cent survival and the entire series a 54 per cent survival. The results in general confirm the earlier studies of Winslow and Cohen (1918) and indicate that the strain of *Bact. coli* studied persists in distilled water for twenty-four hours with only slightly diminished numbers at a pH of 6.0, but that it shows a sharp reduction in solutions which are more acid than 5.0 or more alkaline than 7.0.

TABLE 2
Viability of Bact. coli in distilled water after nine hours

	pH						
	4.0	5.0	6.0	7.0	7.5	8.0	x
Per cent of bacteria surviving.....	1	82	106	54	35	12	89

It will be noted that the viability for unregulated (and unknown) pH values (in the column headed "x") corresponds approximately to the results obtained at a regulated pH of 6.0, showing little or no decrease in 9 hours. We have reason to believe that the solutions used in these early tests were initially distinctly alkaline (following the addition of bacteria); with a pH in the neighbourhood of 8.0, which would have been expected to show a marked reduction in numbers. The fact that an unregulated alkaline solution gives the same results as a regulated neutral solution, as will be explained in a succeeding paragraph, is due to the buffering properties of the bacterial cells or of their products in solutions whose hydrogen-ion concentrations are not artificially controlled.

Our next problem was to determine the influence exerted upon the viability of our test organism by a salt with a monovalent and one with a bivalent cation. For this purpose we chose the chlorides of sodium and calcium.

THE EFFECT OF SODIUM CHLORIDE IN DIFFERENT CONCENTRATIONS
AND AT VARIOUS ACIDITIES

To study the influence of sodium chloride upon the viability of *Bact. coli* we suspended the organisms in salt solutions of varying strengths, from 0.0145 molar (0.1 isotonic) to 1.45 molar (10 isotonic). The technique of manipulation was otherwise the same as that previously described. At the beginning of our work we made up our solutions in fractions and multiples of isotonic strength, following the example of Loeb and other students of the higher organisms. It seems doubtful, however, how far the conception of isotonicity applies to the bacterial cell; and in order to avoid any a priori assumptions we have expressed our data in the present paper in molar terms. For comparability we have, however, kept the same actual concentrations throughout, in spite of the fact that when expressed in molar terms the figures appear as irregular fractions.

Tables 3 to 8 present our findings from 99 experiments in which various concentrations of sodium chloride were studied through twenty-four hour periods under different pH conditions. The figures in table 3 for 0.0145 and 0.0725 molar solutions of sodium chloride show an unquestionably favorable effect on viability. By comparison with the data in tables 1 and 2 which show the mortality in distilled water it is seen that after any incubation period the per cent of bacteria surviving is greater in these salt solutions than in water even at the most favorable pH (6.0). Similarly, table 4 shows that viability is favored in 0.145 molar sodium chloride solution. Even in the four test solutions incubated at pH 5.0 to 6.0 there were still 100 or more per cent of the bacteria alive after 9 or 24 hours as compared with 82 to 77 per cent alive at the favorable pH 6.0 in water. On the other hand a 0.435 molar NaCl solution in one experiment (table 5) conducted at pH 7.3-7.6, was distinctly unfavorable for the survival of *Bact. coli*, and in six experiments in which pH was not regulated a very slight toxicity was manifest, the reduction in numbers, however, being scarcely greater than in distilled water with unregulated reaction. The averages of these 7

TABLE 3

Viability of Bact. coli in sodium chloride solution; 0.1 isotonic (0.0145 M) and 0.5 isotonic (0.0725 M)

HOURS	PER CENT ALIVE	
	0.0145 M—pH not known	0.0725 M— 1 experiment pH not known, 1 experiment pH 7.0
1	100	91
3	105	96
6	77	121
9	115	86
24	140	82
Number of experiments.....	7	2

TABLE 4

Viability of Bact. coli in sodium chloride solution; 1.0 isotonic (0.145 M)

HOURS	PER CENT ALIVE			
	pH 5.0—6.0	pH 7.0—7.4	pH not known	All pH values
1	75	90	97*	88*
3	85	91	105	97
6	81	71	101	91
9	101	76	83*	82*
24	<u>114</u>	80	<u>89</u>	<u>84</u>
Number of experiments.....	4	2	10	16

* One unusually high figure omitted from average.

TABLE 5

Viability of Bact. coli in sodium chloride solution; 3 isotonic (0.435 M)

HOURS	PER CENT ALIVE		
	pH 7.3—7.6	pH not known	All pH values
1	<u>94</u>	109	107
3	57	68	66
6	7	71	<u>61</u>
9	0.4	64	55
24	0	<u>63</u>	54
Number of experiments.....	1	6	7

TABLE 6

Viability of Bact. coli in sodium chloride solution; 5 isotonic (0.725 M)

HOURS	PERCENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	Unknown
1	0+	6	82	101	94	98	67	85
3	0+	0.4	78	72	71	38	52	67
6		0.1	54	24	57	26	21	49
9		0.2	43	12	33	11	9	46
24		0.1	13	1	5	0+	0+	30
Number of experiments...	1	4	6	2	10	3	3	18

TABLE 7

Viability of Bact. coli in sodium chloride solution; 6 isotonic (0.870 M)

HOURS	PERCENT ALIVE AT pH				
	6.0	6.5	7.0	7.5	8.0
1	86	86	23	54	55
3	15	35	1	6	6
6	11	6	0.2	0.4	0.2
9	1	1	0+	0+	0+
24	0+	0+	0+	0+	0+
Number of experiments...	A single experiment				

TABLE 8

Viability of Bact. coli in sodium chloride solution; 7 isotonic (1.015 M) and 10 isotonic (1.450 M)

HOURS	PERCENT ALIVE—pH NOT KNOWN	
	1.015 M	1.450 M
1	91	61
3	51	16
6	33	1
9	20	0+
24	8	0+
Number of experiments.....	9	10

experiments indicate that sodium chloride is probably slightly toxic in a 0.435 molar concentration. From the results of 47 experiments with sodium chloride in 0.725 M concentrations summarized in table 6 it appears that a fairly marked toxicity has been attained. When sodium chloride solution exceed 0.725 M concentrations the toxicities of the solutions increase rapidly. In the single experiment conducted with 0.870 molar concentration (table 7) the greatest viability was evidenced at pH 6.0 and 6.5 although the rate of mortality was at all points very great. Concentrations of 1.015 M and 1.450 M (table 8) are uniformly of marked toxicity, killing 80 to 100 per cent of the bacteria in nine hours (table 8).

Throughout all of this work we have found that when measuring viability in non-toxic concentrations of NaCl or in water an occasional test solution will show a decided toxicity not manifested by others of similar strength. Such variations are lost sight of when the data are tabulated in terms of averages as we have done. Lack of space forbids presentation of the individual findings in the hundreds of tests which we have conducted or even a brief discussion of the hypotheses upon which these experimental divagations may be explained. Fantus and Rumry (1920) have demonstrated that neither concentration of inoculum nor presence or absence of clumps is responsible for such variations. With solutions of molar strength the factor of variability appear to be almost completely abolished. In these concentrations NaCl is uniformly and markedly toxic. In general it seems to us that our experiments are sufficiently numerous to warrant the general conclusion that a 0.14 M or less concentration of sodium chloride is favorable to the survival of *Bact. coli*; that a 0.435 M concentration is without marked toxic action upon the bacterium at all pH values studied; that 0.725 M sodium chloride possesses a distinct harmful effect which is least marked in the zone pH = 6.0 to 7.0; and that solutions over a molar strength are powerfully toxic.

Inasmuch as the 99 tests with NaCl which are summarized in tables 3 to 8 were conducted over a period of about three and one-half years, we thought it wise to repeat the series in a single

experiment, in order to determine how accurately we could check the outstanding findings on the relation between concentration and toxicity of this earlier work. We seeded 40 test solutions from a single suspension of *Bact. coli* nineteen hours old in distilled water, kept in an ice bath during the seeding period. The solutions had been sterilized by boiling, instead of in the autoclave, to avoid contamination with ammonia and other volatile substances commonly present in the steam of sterilizers. Before seeding, the solutions had pH values of 8.4 to 8.6, and immediately after seeding with about 35,000,000 *Bact. coli*

TABLE 9

Viability of Bact. coli in various concentrations of sodium chloride

SOLUTION— CONCENTRATION NaCl	SURVIVAL OF BACTERIA AFTER NINE HOURS					
	Experiment 98		All previous experiments		All experiments	
	Per cent alive	Number of tests	Per cent alive	Number of tests	Per cent alive	Number of tests
0	95	5	89	11	91	16
0.0145 M	121	5	115	7	118	12
0.0725 M			86*	2	86*	2
0.145 M	93	5	83	10	86	15
0.435 M	70	5	64	6	67	11
0.725 M	56	5	46	18	48	23
0.870 M	33	5			33	5
1.015 M	18	5	20	9	19	14
1.305 M	6	5			6	5
1.450 M			1	10	1	10

* This figure is introduced to make the tabulation complete. Inasmuch as it is the average of only two tests its validity is perhaps questionable.

per cubic centimeter, of 8.4 to 8.7. For each concentration of sodium chloride there were 5 test solutions. The results of this experiment (Experiment 98) are summarized in table 9 where they are compared with the summarized data of all comparable previous experiments (64 in number) in which viability in sodium chloride was tested and in which there were no adjustments or readjustments of pH.

The unusually close checks obtained renewed our confidence in the accuracy of the results obtained. We may safely conclude that the summarizing column "All experiments" in table 9

describes with reasonable accuracy the viability of *Bact. coli* in solutions of sodium chloride of varying concentration in which no adjustment of pH is made during the course of the test.

When we compare these results, shown in table 9, obtained at unregulated and unknown pH values (but with solutions starting at a pH in the neighborhood of 8.0 or over) with those obtained at a pH value adjusted during the course of the experiment, we find that as with distilled water the unadjusted alkaline solutions give results comparable with those obtained at a regulated pH of about 6.0, (see tables 4, 5, and 6).

EFFECT OF CALCIUM CHLORIDE IN DIFFERENT CONCENTRATIONS AND AT VARIOUS ACIDITIES

Table 10 shows the results obtained with CaCl_2 solutions of less than M/10 strength. With the three more dilute solutions (0.00145 M 0.0145 M, 0.029 M) there was the usual variation between parallel bottles always manifest in non-toxic solutions, one bottle showing a decrease, while its companion showed a marked increase. The average results for all the concentrations mentioned, however, show a distinctly higher viability than is manifest in distilled water (96 per cent or more alive after twenty-four hours). These low concentrations, as in the case of NaCl seem to be definitely favorable to viability.

A concentration of 0.072 M CaCl_2 on the other hand seems to show toxic action; and with a solution of 0.145 M strength we found an even clearer toxicity, at least *in those solutions in which the pH was not measured or controlled*, (last column of table 11).

The bacteria hold their own for the first hour but after that decrease rapidly. After two to three hours only 4 bottles out of 19 in one group, for example, contained over 15 per cent of their original numbers and after twenty-four hours 15 out of 18 bottles showed 1 per cent or less of their original germ content, while the other 3 showed 3 per cent, 10 per cent, and 36 per cent, respectively. For a total of 32 tests at unregulated pH we found 70, 40, 27, 22 and 16 per cent alive after one, three, six, nine and twenty-four hours, respectively. The results obtained with adjusted acidities will be discussed in a succeeding paragraph.

Concentrations of calcium chloride of 0.435 M strength and over showed at all reactions a still more rapid and uniform toxic effect than that indicated for the 0.145 M solution in table 11. Nine tests were made with 0.435 M solutions (table 12). In 6 out of the 9 less than 1 per cent of the bacteria remained after three

TABLE 10

Viability of Bact. coli in calcium chloride solutions; 0.01 isotonic (0.00145 M), 0.1 isotonic (0.0145 M), 0.2 isotonic (0.029 M), 0.5 isotonic (0.072 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN			
	0.00145 M	0.0145 M	0.029 M	0.072 M
1		73	94	88
3	92	84	89	51
6	158	64	105	41
9	166	70	92	45
24	152	96	110	37
Number of experiments.....	2	20	3	5

TABLE 11

Viability of Bact. coli in calcium chloride solutions: 1 isotonic (0.145 M)

HOURS	PER CENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	Un-known
1	42	79	120	127	84	70	86	70
3	1	9	120	108	75	87	99	40
6	0+	2	124	146	66	58	38	27
9	0+	1	78	109	71	61	31	22
24	0	0+	90	61	49	31	12	16
Number of experiments....	1	1	3	2	9	2	2	32

Buffered solutions not included.

hours (the other 3 bottles showing a survival of 19 per cent, 32 per cent and 28 per cent at this period). After six hours these 3 bottles in which the resistance was relatively high showed 2 per cent, 7 per cent and 3 per cent respectively and after nine hours, 0.1 per cent, 1.9 per cent and 0.2 per cent.

Ten tests were made in 0.725 M solutions with the following results. In 5 bottles less than 1 per cent of the bacteria survived after three hours (the per cent surviving in the other 5 bottles being 19 per cent, 3 per cent, 4 per cent, 4 per cent and 9 per cent, respectively). After six hours all had dropped to 1 per cent or less. Five tests in 1.015 M calcium chloride showed less than 1 per cent surviving after three hours in 4 bottles, 1 per cent and 4 per cent in the other 2 cases (table 12).

TABLE 12

Viability of Bact. coli in calcium chloride solutions: 3 isotonic (0.435 M), 5 isotonic (0.725 M), 7 isotonic (1.015 M), 10 isotonic (1.450 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN				
	0.435 M		0.725 M	1.015 M	1.45 M
	pH=7.6				
1	64	37	25	0+	15
3	28	9	4	0+	2
6	3	1	0.4	0	0+
9	0.2	0+	0+	0	0+
24	0	0+	0+	0	0
Number of experiments..	1	9*	10	5	6

* Includes one experiment with pH unknown.

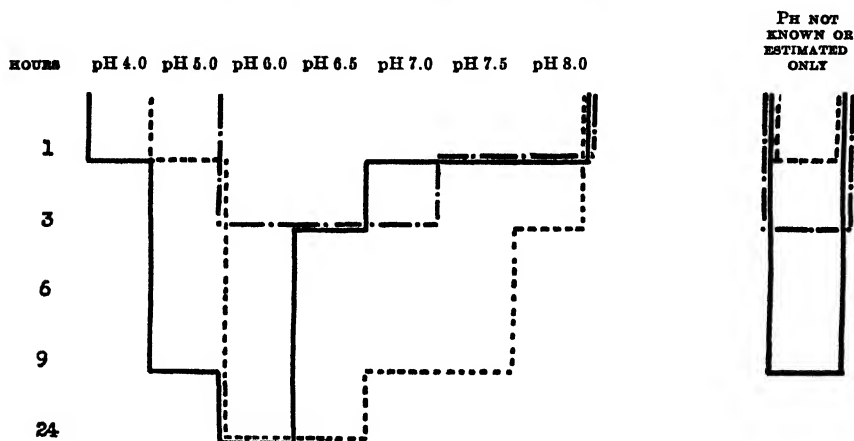
EFFECT OF REACTION IN THE PRESENCE OF SODIUM AND CALCIUM SALTS, RESPECTIVELY

We come now to a consideration of the somewhat puzzling results indicated by table 11 in regard to the influence of the reaction of the calcium chloride solutions upon viability. It has been pointed out above that, in distilled water and in the presence of NaCl, unregulated alkaline solutions (starting in the neighborhood of pH 8.0) show a high degree of viability similar to that obtained at an adjusted reaction of about pH 6.0; and this has been explained as probably due to reversion to a neutral reaction taking place in the presence of the bacterial cells. Yet with CaCl₂ the results observed in an unadjusted alkaline solution instead of corresponding with those obtained at a regulated pH of 6.0 show a high toxicity comparable with that characteristic of a regulated pH 8.0.

Tables 13 and 14 show very clearly the apparent conflict in the results observed. These tables include a number of special experiments conducted to cover the nine hour period alone in

TABLE 13

The relation between viability of Bact. coli and pH in distilled water, 5.0 isotonic NaCl (0.725 M) and 1.0 isotonic CaCl₂ (0.145 M) solutions



Lines indicate times and reactions at which one-third of the bacteria were dead.

————— Viability in distilled water.

----- Viability in 5.0 isotonic NaCl.

..... Viability in 1.0 isotonic CaCl₂.

TABLE 14

Effect of pH upon viability of Bact. coli in water, 5.0 isotonic (0.725 M) NaCl and isotonic (0.145 M) CaCl₂

SOLUTION	PER CENT OF BACTERIA SURVIVING AFTER NINE HOURS						
	5.0	5.5	6.0	7.0	7.5	8.0	\bar{x}
Water.....	82		106	54	35	12	89
5.0 isotonic NaCl.....	27	20	87.	76	8	9	46
1.0 isotonic CaCl ₂	134		128	106	44	31	22

order to eliminate chance variations by obtaining averages based on a large number of observations. In these tables we have omitted all determinations not based on at least four tests. The results

differ at certain points from those presented in tables 1, 6 and 11 but where this is the case the results in the latter tables were based on 1 or 2 tests only. The bases for tables 13 (136 tests) and 14 (about 250 tests) are sufficiently broad to be reasonably reliable. In water and NaCl solutions the results obtained at an unadjusted alkaline pH correspond approximately to those observed at an adjusted pH of 6.0 or 7.0. In CaCl_2 solutions, on the other hand, the results obtained at an unadjusted alkaline pH correspond to those observed at an adjusted pH of about 8.0. Thus at an adjusted pH of 6.0, or at an adjusted pH of 8.0 or at any other adjusted pH, 0.725 M NaCl is slightly more toxic than

TABLE 15

Viability of Bact. coli in 0.145 M calcium chloride with hydrogen ion concentration increased by carbon dioxide from the breath

INCUBATION PERIOD <i>hours</i>	AVERAGE PER CENT SURVIVING	
	pH decreased by CO_2 from breath (average of 7 tests)	No CO_2 (Average of 32 tests— table 12)
1	139	70
3	114	40
6	75	27
9	67	22
24	52	16
120	3	

0.145 M CaCl_2 , in an unadjusted alkaline solution on the other hand 0.145 M CaCl_2 is distinctly more toxic than 0.725 M NaCl.

This particular concentration of CaCl_2 (0.145 M) seems then to display its characteristic toxicity only in an unregulated alkaline solution. The importance of the reaction factor was strikingly called to our attention by the high degree of viability manifest in certain tests in which the suspensions were stirred by blowing into them through the sampling pipettes. Table 15 shows how the carbon dioxide introduced in this way tended to abolish the toxic effect of 0.145 M CaCl_2 .

An explanation of these phenomena has apparently been found by following out the observations made two years ago (Falk, 1920) as to the power of bacterial suspensions to regulate the pH

of their menstruum and the influence of salts upon that power. These earlier results warranted the assumption that in our experiments in which pH was uncontrolled the initial pH (in each case, an alkaline one) had remained alkaline in the CaCl_2 solutions but had shifted towards the neutral point (7.2) in the water and NaCl solutions. We tested this hypothesis in a series of 5 experiments (93 to 98) in which 50 test solutions were studied. We have summarized these results, which have been already reported in preliminary fashion (Winslow and Falk, 1922) in table 16.

It appears that at a pH controlled at about 6.0 neither water nor 0.145 M CaCl_2 has toxic action; that in water with an initial pH over 9.0 the reaction falls to about 8.0 in nine hours and no toxic effect is manifest; and, finally, that in a 0.145 M CaCl_2 solution of an initial pH over 9.0 the reaction changes but

TABLE 16

Change in pH and viability of Bact. coli in water and 1.0 isotonic (0.145 M) CaCl_2

SOLUTIONS	DISTILLED WATER			ISOTONIC CaCl_2 (0.145 M)		
	pH initial	After nine hours	Per cent bacteria alive after nine hours	pH initial	After nine hours	Per cent bacteria alive after nine hours
Unadjusted pH.....	9.2	8.0	91	9.2	8.9	1
Continuously adjusted pH.....	6.0	6.1	76	6.0	6.5	90

slightly and the bacteria show the high mortality to be expected in such an alkaline solution. It therefore appeared to us that the apparent conflict between the toxicities, absolute and relative, of the test solutions when pH was controlled and when it was uncontrolled, was explicable on the ground that although the unregulated water and sodium solutions on the one hand and calcium solutions on the other may begin an incubation period at the same pH, they do not have the same pH throughout the period. And, hence, the toxicities which are found in such solutions are not comparable with the relative toxicities of solutions kept by careful readjustment at a constant pH.

There remained the question whether the difference in reaction changes was quantitatively adequate to account for the observed differences in viability. The alkalinity of the unadjusted water

solution falls considerably it is true, but does it fall enough to account for the high viability displayed? Thus it appears in table 16 that a pH of 8.0 after nine hours (initial pH = 9.2) is non-toxic while, as previously indicated, a pH *maintained* at 8.0 throughout an experiment is highly toxic. It occurred to us that we might explain this apparent anomaly on the assumption that the regulating action of the bacteria operates in zones immediately surrounding the bacterial cells much more effectively than in the main bulk of the suspending fluid. If therefore the fluid is not stirred frequently or vigorously the bacteria in a solution which they are thus regulating may actually be exposed to a reaction much nearer neutrality than is indicated by a pH reading of the test solution as a whole. In such a case our pH readings do not represent the actual concentration of hydrogenions about the cell as they do in a suspension in which reaction is being controlled artificially by repeated shaking, examination of aliquot samples and readjustment of pH.

If this explanation is correct, it should follow that in bacterial suspensions whose pH is not being controlled by readjustments the shift in general pH should be greater in stirred than in un-stirred bottles. That is, if in an alkaline suspension the bacteria secrete acidic substances (acidic relative to the pH of the suspensions) the pH of the suspension as a whole will remain comparatively unchanged if for lack of stirring of the solution these acidic substances remain in immediate contact with the bacteria, on the assumption that these acidic substances are secreted only as long as the menstrua are alkaline in the zone about the cell. On the other hand, if the solutions are stirred frequently the neutralizing substances secreted by the cells into the alkaline menstuum will be evenly distributed throughout the solution; the bacteria will be repeatedly exposed to a comparatively alkaline reaction and thus should be induced to secrete more and more of the neutralizing substances.

In order to test this assumption we prepared six bottles of each of the following solutions—water; 0.01, 0.1, 1.0, 3.0, 5.0, 7.0 and 10.0 isotonic NaCl; and 0.01, 0.1, 0.5, 1.0 and 3.0 isotonic CaCl₂ (isotonic solution is 0.145 M). All the bottles were set out

on a table where they would not be disturbed and were seeded from a single suspension of *Bact. coli* in distilled water so that each would contain approximately 30,000,000 bacteria per cubic centimeter. The pH of each was read after seeding and adjusted, if necessary, to 8.5 to 8.7. Each group of 6 bottles was then separated by random choice into two groups of 3 bottles each, one group for the "shaken" and the other for the "unshaken" tests. After three, six, nine and twenty-four hours' incubation,

TABLE 17

Effect of mechanical agitation upon change in pH. Suspensions of Bact. coli in water and in NaCl and CaCl₂ solutions

SOLUTION— MOLAR CONCENTRATION*	pH CHANGE (INITIAL pH = 8.5–8.7) AFTER INCUBATION FOR							
	Three hours		Six hours		Nine hours		Twenty-four hour	
	Shaken	Not shaken	Shaken	Not shaken	Shaken	Not shaken	Shaken	Not shaken
Water	0.2	0.0	0.9	0.1	1.2	0.2	1.3	0.8
NaCl								
0.00145	1.1	0.7	1.1	1.0	1.2	1.1	1.2	1.2
0.0145	1.1	0.5	1.2	1.2	1.3	1.3	1.3	1.3
0.145	0.4	0.2	1.2	0.9	1.3	1.0	1.2	1.1
0.435	0.3	0.0	1.0	0.1	1.1	0.3	1.2	0.9
0.725	0.3	0.0	1.1	0.1	1.0	0.2	1.1	0.9
1.015	0.7	0.0	0.9	0.1	0.9	0.2	1.2	0.8
1.450	0.0	0.0	0.0	0.0	0.2	0.2	0.9	0.5
CaCl ₂								
0.00145	0.3	0.0	0.3	0.1	0.4	0.2	1.3	0.5
0.0145	0.4	0.2	0.7	0.2	0.9	0.3	1.2	0.6
0.0725	0.1	0.0	0.6	0.0	0.6	0.1	0.6	0.6
0.145	0.1	0.1	0.2	0.2	0.5	0.2	0.5	0.2
0.435	0.1	0.0	0.2	0.0	0.2	0.0	0.2	0.1

* 1 tonicity = 0.145 M.

pH samples were taken from each bottle. On these occasions, the "shaken" bottles were stirred vigorously before an aliquot was withdrawn. (They were also stirred at one-half hour intervals during the first nine hours between pH readings.) The "unshaken" bottles were never handled and were watched with great care to avoid accidental disturbance. Fine-tipped pipettes were used, and the samples were withdrawn slowly and carefully to avoid stirring and backwash. All of the test solutions were

allowed to become saturated with air before the experiment was begun. For the sake of convenience in manipulation, the work was done at room instead of incubator temperature. The findings are summarized in table 17 where each figure is the average of 3 determinations.

The uniformity of the results in this experiment was very striking. It seems clear that in alkaline solutions the pH tends to shift towards a neutral zone (pH 7.3 to 7.4); that it shifts more rapidly in bacterial suspensions which are being shaken than in those which are not being shaken; that the pH change is greater or more rapid in the less toxic solutions; and that the shift is greater and more rapid in solutions of NaCl than in solutions of CaCl_2 of approximately the same toxicity. These results completely confirm our hypotheses and, it appears to us, explain the apparently anomalous results described above. It is evident that relatively slight differences in the general pH of a solution under such conditions must indicate very great differences in the restricted pH of the zones immediately surrounding the bacterial cells.

It appears then that the toxic effect of 0.145 M CaCl_2 is an indirect one and is exerted only in an alkaline solution in which the salt interferes with the regulative action exerted by bacterial cells upon the reaction of such a solution.

In his studies on the phenomenon of agglutination Buchanan (1919) has pointed out that under appropriate conditions and in certain concentrations CaCl_2 serves as an agglutinating agent with suspensions of meningococci. This suggests that the apparent reduction in numbers of bacteria in CaCl_2 solutions may be attributable to agglutination rather than to true toxicity. Although there may be as many viable cells in an agglutinated suspension as in a non-agglutinated one, the number of colonies appearing on an agar plate will be different in the two cases. Direct microscopic observations of CaCl_2 suspensions of *Bact. coli*, however, indicate that with the conditions under which our experiments were conducted agglutination did not ordinarily occur. The rôle of the H-ions present in their influence on agglutinability has not yet, however, been thoroughly studied in this connection.

SUMMARY AND CONCLUSIONS

1. The strain of *Bact. coli* with which we have conducted our experiments maintains itself in distilled water at a favorable pH value without material decrease in numbers for a period of nearly twenty-four hours. Increases are not uncommon during the first few hours. Occasionally however a particular suspension will show a marked decrease due to some cause which we have not yet determined.

2. A reaction of about pH 6.0 is most favorable to the viability of these bacteria in distilled water, the viability decreasing as a solution becomes more acid or more alkaline.

3. A NaCl solution of 0.0145 M strength exerts a distinctly favorable action upon viability. Instead of a slight but definite decrease after twenty-four hours we find that in these highly dilute salt solutions the bacteria maintain themselves in undiminished numbers. The same favorable result is apparent in a CaCl_2 solution of 0.00145 M strength.

4. NaCl solutions of 0.725 M strength and over and CaCl_2 solutions of 0.435 M strength and over are distinctly toxic at all reactions.

5. Finally a CaCl_2 solution of intermediate strength (0.145 M) shows very peculiar and interesting results. At any pH value maintained throughout an experiment by repeated readjustments such a solution is non-toxic. In an unadjusted alkaline solution, however, it displays a definite toxic action which we have demonstrated to be due to the fact that the CaCl_2 prevents the bacteria from bringing about a reversion to a more favorable neutral reaction. Our experiments make it clear that in an alkaline solution the bacteria normally alter the medium in the zone immediately adjacent to them in such a way as to decrease its alkalinity to a very marked degree, a process which is inhibited by 0.145 M CaCl_2 .

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STUDIES ON SALT ACTION

IX. THE ADDITIVE AND ANTAGONISTIC EFFECTS OF SODIUM AND CALCIUM CHLORIDES UPON THE VIABILITY OF BACT. COLI

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In the immediately preceding paper of this series (Winslow and Falk, 1923) we have presented in some detail the results of our studies upon the influence of two salts with a common anion, one with a typical monovalent and one with a typical divalent cation, upon the viability of *Bact. coli* in water. It was there shown that at any pH value 0.725 M NaCl and 0.435 M CaCl₂ solutions are toxic to this organism and that 0.145 CaCl₂ is toxic in an alkaline solution (pH 8.0), its toxicity here being due to the fact that CaCl₂ under such conditions inhibits the ability of the bacteria to regulate the reaction of the solution.

Over four years ago (Winslow and Falk, 1918) we presented a preliminary report which indicated that in a solution containing sodium and calcium salts in appropriate proportions an antagonistic action may be manifest which tends to protect the bacteria against the toxic action which would be exerted by each salt if present alone. The experiments here reported deal with this question, the technique being the same as that reported in the paper first cited (Winslow and Falk, 1923).

A solution of calcium chloride of 0.145 M strength, the strength at which toxic action first appears in an unadjusted alkaline solution, was chosen for the study of antagonism and in table 1 are presented the results of 21 tests in solutions of calcium chloride of this strength plus various concentrations of sodium chloride. The solution was made up alkaline (about pH 8.0) but was not adjusted thereafter and changes in reaction were not recorded.

In this table we have followed the procedure employed in the previous paper and have drawn a heavy rule to indicate the time after which less than approximately two-thirds of the initial bacterial population are still viable. Table 1 shows that a mixture of NaCl and CaCl₂ is moderately toxic when the two are present in equal proportion and each in 0.145 molar ("isotonic") concentration, only 38 per cent of the bacteria surviving six hours or longer. (This strain of *Bact. coli* survives in nearly undiminished numbers—89 per cent—for nine hours' in pure, distilled water.) It shows, further, that the toxic effects of the

TABLE 1

Viability of Bact. coli in solutions containing sodium chloride + calcium chloride

		Per cent surviving				
		PERCENT ALIVE				
NaCl + CaCl ₂		1 + 1	2 + 1	3 + 1	4 + 1	5 + 1
Tonicity*.....		2	3	4	5	6
1		114	65	124	90	165
3		80	52	55	110	89
6		38	27	38	104	44
9		41	28	40	117	30
24 hours		35	28	22	90	0+
Number of experiments ..		8	3	3	4	3

* 1 tonicity = 0.145 M.

salts are additive, since solutions which contain the same quantity of CaCl₂ (0.145 mol per liter) but two and three times as much NaCl (2 and 3 × 0.145 mols per liter) are increasingly toxic. When 4 × 0.145 molar concentration of NaCl is added to 1 × 0.145 molar concentration of CaCl₂, however, the toxicity instead of increasing further with the increase in total concentration of the solution diminishes very markedly. In this 5 "isotonic" solution over 100 per cent of the initial number of bacteria are still alive after nine hours as compared with 28 to 41 per cent for a similar incubation period in solutions containing the same

quantity of CaCl_2 but lesser quantities of NaCl . Solutions containing 6×0.145 mols per liter ($5 \text{ Na} + 1 \text{ Ca}$) become more toxic again but remain less toxic than $3(2 \text{ Na} + 1 \text{ Ca})$ or $4(3 \text{ Na} + 1 \text{ Ca})$ times isotonic solutions up to six hours' exposure. In other words, we may state from these experiments, that the toxic influences of NaCl and CaCl_2 upon the viability of *Bact. coli* in water are additive up to a certain ratio of $\text{Na}:\text{Ca}$ after which they become antagonistic.

The additive and antagonistic influences of NaCl and CaCl_2 are summarized in somewhat more convenient form in table 2 and in figure 1 where they are presented with the results from

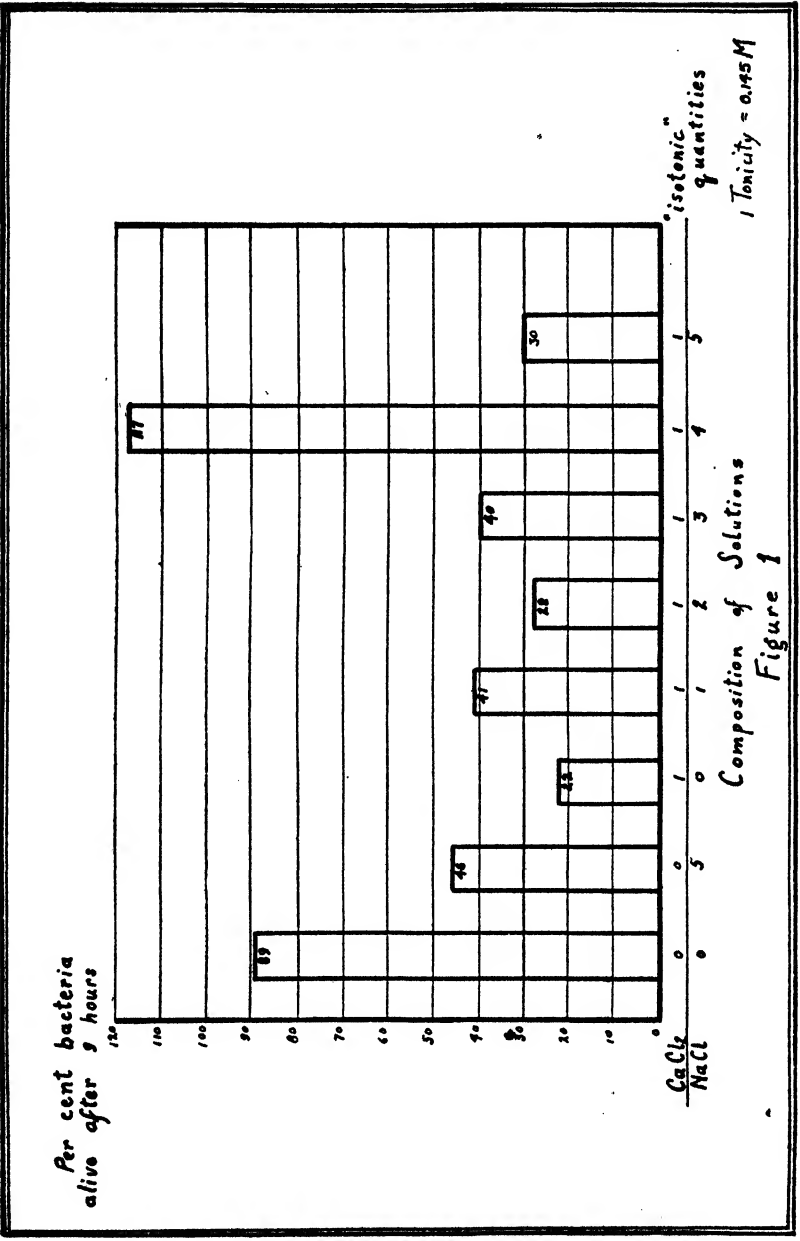
TABLE 2

Viability of Bact. coli in solutions of NaCl and CaCl₂, singly and in combinations

TOTAL ISOTONIC CONCENTRA- TION*	TOTAL MOLAR CONCENTRA- TION	PER CENT BACTERIA ALIVE AFTER NINE HOURS IN				NUMBER OF EXPERIMENTS AVERAGED		
		Pure NaCl	Pure CaCl_2	$\text{NaCl} +$ CaCl_2	Ratio $\text{Na}:\text{Ca}$	NaCl	CaCl_2	$\text{NaCl} +$ CaCl_2
0	0	89	89	89		11	11	11
1	0.145	82	22			16	32	
2	0.290			41	1:1			8
3	0.435	55	0+	28	2:1	7	9	3
4	0.580			40	3:1			3
5	0.725	46	0+	117	4:1	18	10	4
6	0.870	33		30	5:1	5		3

* 1 tonicity = 0.145 M.

comparable experiments with the pure salts used singly. They indicate clearly that in appropriate proportion (4:1), NaCl and CaCl_2 are mutually antagonistic to each other with respect to toxicity towards *Bact. coli*. In this proportion and in an absolute concentration in which NaCl by itself is moderately toxic (46 per cent of the bacteria surviving nine hours at 37°C .) and in which CaCl_2 is highly toxic (5 iso CaCl_2 often produces sterility in nine hours and uniformly reduces the concentration of viable bacteria by more than 99.5 per cent) the mixture of NaCl and CaCl_2 permits the bacteria to survive in undiminished numbers for nine to twenty-four hours. Indeed, after nine hours there were still 117 per cent of the bacteria alive as compared to 89 per cent in pure water.



In the experiments presented thus far no attempt was made to modify or control the pH of the solutions. In a preliminary publication (Falk, 1920) and in the preceding paper in this Journal (Winslow and Falk, 1923) we have indicated certain findings in regard to the influence of hydrogen-ion concentration upon the action of these salts. We have shown that the viability of *Bact. coli* in pure solutions of water, of NaCl and of CaCl₂ is greatest at pH 6.0 to 6.5 and diminishes rapidly at a smaller pH and more slowly at a greater pH. The results of 19 experiments on the influence of pH (maintained by repeated readjustment)

TABLE 3

Viability of Bact. coli in solutions containing sodium chloride + calcium chloride

NaCl + CaCl ₂	PERCENT ALIVE						
	5 + 1 (tonicity = 6)*						
pH.....	4.0	5.0	6.0	6.5	7.0	7.5	8.0
1	0+	74	88	106	98	92	90
3	0+	86	82	100	84	80	75
6	0+	62	73	92	71	57	54
9	0+	0.2	71	62	50	45	41
24 hours	0+	0+	24	21	13	1	9
Number of experiments	1	1	2	3	6	2	4

* 1 tonicity = 0.145 M.

upon viability in solutions of the two salts, NaCl and CaCl₂, are summarized in tabular form in table 3, while in table 4 the nine-hour results are compared with those obtained after the same period in previous experiments by using the same concentration of the salts separately. In the preceding paper of this series we have described in detail the method of adjusting the pH immediately after the bacterial inoculum is added to the test solution and the methods of readjusting the pH if it varied from the stated value by more than 0.2 at stated intervals during the incubation period.

Table 4 and figure 2 bring out the exceedingly interesting fact that at reactions of pH 7.0 and all more acid reactions the effect of NaCl and CaCl₂ is additive, the mixture being more toxic than either salt alone. At regulated alkaline reactions on the other hand (comparable to the experiments with unadjusted reaction reported in tables 1 to 3) the two salts antagonize each other, the mixture being less toxic than either salt alone and markedly less toxic than the added toxicities of the two salts used singly. In addition, it is evident from figure 2 that the toxicity of NaCl, at the strength here studied, appears only at pH values below 6.0 or above 7.0; that of CaCl₂ only above 7.0. It appears also that NaCl *narrows* and CaCl₂ *widens* the zone for hydrogen-ion tolerance of *Bact. coli* and

TABLE 4

Effects on viability of Bact. coli of sodium and calcium chlorides after nine hours' exposure

pH.....	PER CENT BACTERIA ALIVE				
	5.0	6.0	7.0	7.5	8.0
Water.....	82	106	54	35	12
0.725 M NaCl.....	27	87	76	8	9
0.145 M CaCl ₂	134	128	106	44	31
0.725 M NaCl + 0.145 M CaCl ₂	0.2	71	50	45	41

that effects of the salts which are clearly evident in more acid solutions tend to disappear (approach the pure water curve) at the same pH (7.0 to 7.5). Holm and Sherman (1921) and Sherman and Holm (1922) have reported that salts which accelerate growth of *Bact. coli* seem to widen and salts which inhibit growth seem to narrow the zone for H-ion tolerance. It is significant to observe that these effects upon growth are confirmed for viability.

In the preceding paper in this Journal we have called attention to the fact that in water and saline suspensions of *Bact. coli*—regardless of the initial pH—the hydrogen-ion concentration tends to shift to the neutral zone 7.0 to 7.4. This observation has also been reported by Eggerth and Bellows (1922). It is

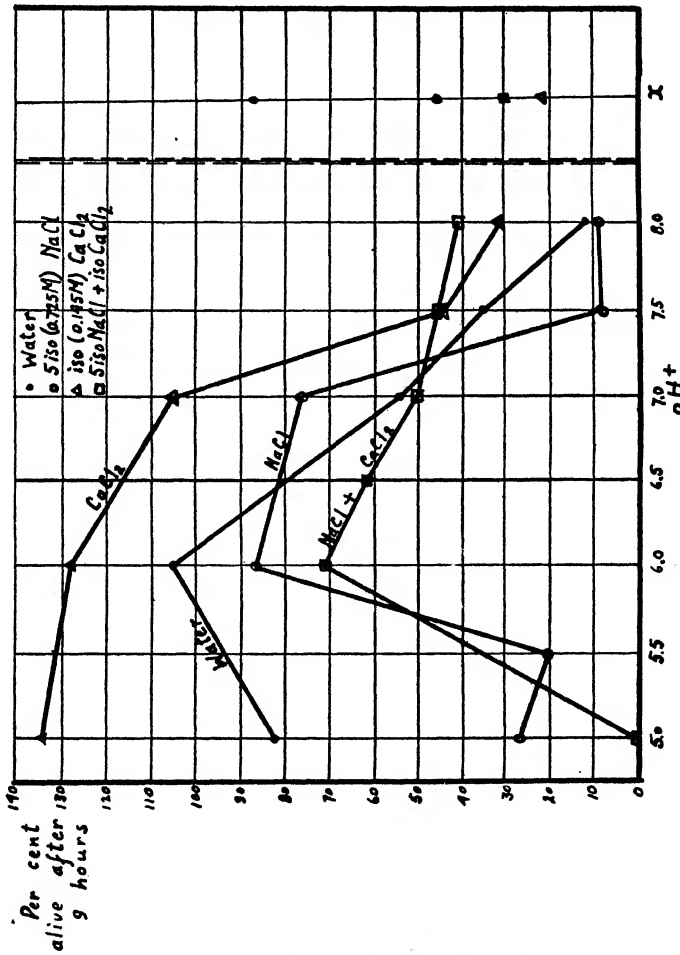


Figure 2

interesting to call attention to the fact that all four curves in figure 2 approach uniform values in this pH zone of maximum physiological buffer. The significance of this observation is being studied in our laboratory.

These results, taken together with those reported in the immediately preceding paper seem to us to warrant the conclusion that the toxic effects exerted by salts may be of two distinct sorts. Very high concentrations of salts appear to exert a toxic effect which is apparent at all reactions and is additive when sodium and calcium chlorides are mixed. At a lower concentration (0.145 M) calcium chloride exerts a different influence, manifest only in alkaline solutions and due to an inhibition of the power of the bacteria to reduce the alkalinity of the solution in which they are suspended. It is this latter type of toxic influence which is antagonized by sodium chloride and in alkaline solution the mixture of these salts in the proportion of 5 parts NaCl to 1 part CaCl₂ is more favorable to viability than even distilled water.

The mechanism of ionic and molecular effects upon bacteria has been discussed by one of us (Falk, 1923) at greater length than is possible here. It is being further studied in this laboratory and will be treated more fully in later papers in this series.

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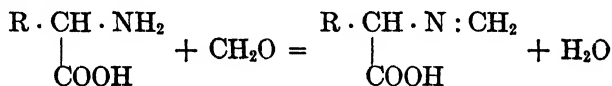
THE FORMOL TITRATION OF BACTERIOLOGICAL MEDIA

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The principles involved in the "formol titration" of bacterial cultures or media are the same as those embodied in the methods of Malfatti (1908) and of Henriques and Sørensen (1909) for the titration of urine. They have been applied to the study of bacterial cultures by a number of authors, notably Kendall, Day and Walker (1913), Itano (1916), Berman and Rettger (1918), Foster and Randall (1921), Ayers, Rupp and Mudge (1921), and Kendall (1922). An excess of formalin (approximately 40 per cent formaldehyde) is added to the sample to be titrated. The sample becomes more acid as a result of the following type reactions:



The increase in acidity is then titrated with a standard alkali solution. Ammonia, primary amines, and the amino groups of amino-acids, and polypeptids react with formaldehyde. The titration, therefore, represents the sum of these substances.

Sørensen (1907) (1908) has pointed out that the reaction of amino-acids with formaldehyde is a reversible reaction, that an excess of formaldehyde tends to throw the reaction, as expressed in the above equation, from left to right, whereas increasing amounts of water throw the reaction from right to left. Since the methylene derivative of the amino-acid is a stronger acid

than the amino-acid and is therefore more easily titrated, it is obviously desirable to force the reaction from left to right. To do this Sørensen found it necessary to add as much as 10 cc. of formalin to 20 cc. of sample (amino-acid solution), and to titrate with N/5 barium hydroxide or sodium hydroxide. Smaller amounts of formalin were insufficient; larger amounts unnecessary. Workers in bacteriology appear not to have appreciated the importance of this factor. Working with solutions of pure amino-acids Sørensen found it necessary to carry the titration to an end point on the alkaline side of pH 8.2 in order to approximate the theoretical value of some of the amino-acids. He therefore titrated to a deep red color with phenolphthalein as an indicator which he states to have been at about pH 9.0, or to a strong blue color with thymolphthalein which he states to have been at about pH 9.7. Sørensen recognized the presence of carbonates and phosphates as a serious source of error in the formol titrations of amino-acid mixtures containing these substances and to minimize this difficulty titrated with barium hydroxide rather than sodium hydroxide, also adding barium chloride to depress the ionization of the barium carbonate and phosphate formed. The methylene derivatives of some polypeptids and certain of the amino-acids, however, notably tyrosine and phenylalanine, formed insoluble precipitates with barium and for their titration better results were obtained with sodium hydroxide. In the methods of Henriques (1909) and of Henriques and Sørensen (1909) the barium carbonate and phosphate were filtered out, the filtrate neutralized, formalin added, and the acid titrated against sodium or potassium hydroxide.

In developing his method Sørensen made formol titrations of pure solutions of many of the amino-acids and of some peptic, tryptic, and ereptic digests of Witte peptone, casein, and egg albumin. Henriques and Sørensen titrated urines. For the titration of bacterial cultures Itano (1916) followed quite closely the method of Sørensen. Kendall and his associates departed widely from the method of Sørensen in that they used a much larger proportion of water (50 cc.) and a smaller proportion of formalin (5 cc.). Foster and Randall (1921) followed the technic

of Kendall, Day and Walker. Berman and Rettger (1918) departed even more widely from the technic of Sørensen in that they used a total volume of 55 cc. of water and only 2 cc. of formalin. Ayers, Rupp and Mudge (1921) presumably followed Sørensen since they do not mention any modification of the technic. Kendall, Day and Walker and Berman and Rettger departed from Sørensen's method in another respect. They "neutralized" their samples of media to the end point of phenolphthalein, a point which Kendall defines as pH 8.3, before adding the formalin. Sørensen (1908) did not do this. He says, after describing the preparation of the control solution to the "deutliche rote Farbe (zweites Stadium)" of phenolphthalein: "Die zur Untersuchung vorliegenden Lösungen werden bis zu dieser letzten Farbenstärke titriert, indem 20 ccm der Analyse 10 ccm Formolmischung zugesetzt werden, und gleich darauf n/5 Barytlauge bis Rotfärbung,"

In the French edition (1907) of his article the meaning is equally clear. He says: "Ensuite on titre jusqu'à la même intensité de coloration les liqueurs à examiner; à cet effet, on ajoute à 20 cc. de la liqueur 10 cc. du mélange de formol et immédiatement après, en agitant bien, la solution de baryte au 1/5, jusqu'à ce que le liquide devienne rouge,"

It seems worth while quoting the above passages verbatim because the description of Sørensen's method in certain text books of physiological chemistry would lead one to believe that the solution to be analyzed should be titrated to the color of phenolphthalein produced in the second stage of the control before the formalin is added.

For the titration of solutions of pure amino-acids Sørensen (1907) (1908) apparently carried out no preliminary neutralization or adjustment of hydrogen ion concentration, though when it was necessary to add alkali to get the substance into solution (e.g. tyrosine and aspartic acid) the amount of alkali added was considered in the computation of the formol titration. Later Henriques and Sørensen (1909) (1910) pointed out that the neutral point of the amino-acids is at about pH 6.8 and that this therefore should be the starting point for the titration. They

brought their samples to this reaction by means of a specially prepared litmus paper. Northrop (1921) adopted pH 7.0 with neutral red as an indicator for the starting point of the titration of hydrolyzed gelatin. H. Jessen-Hansen in Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*, vi, 262, gives a very good discussion of the importance of this factor. Theoretically the isoelectric point should be the point of departure for the titration of amino-acids. Fortunately for our purpose the isoelectric points of the various amino-acids are so close together that their isoelectric zones (Michaelis, 1914, p. 40) overlap and pH 6.8 or 7.0 may be chosen as the ideal starting point for the titration of mixtures of amino-acids and ammonium salts.

The experiments and curves which follow serve to illustrate the importance of choosing the correct hydrogen ion concentrations for the initial and end points of the formol titrations. In bacteriological culture fluids we encounter not only mixtures of amino-acids and ammonium salts but also peptones, polypeptids, fatty acids, carbonates, phosphates, and carbohydrates. The reaction may be acid or alkaline. There are also the disturbing factors of color and turbidity. By known methods of precipitation, filtration, etc. it is possible to eliminate the disturbing factors in such a mixture and to make an accurate formol titration. The present paper is an attempt to analyze the factors involved and to reduce the formol titration of bacteriological media and cultures to its simplest terms.

Phenol red or thymol blue have been used as indicators. The end point is judged by comparison with colorimetric hydrogen ion standards in a comparator block. This not only leads to much greater accuracy in judging the end point but makes it possible to use a color screen of the material being titrated, doing away with the necessity for using artificial colors in a control as was done by Sørensen. Since this work has been in progress Northrop (1921) has also made similar use of the comparator block and colorimetric standards. The titration is carried on in large test tubes containing 1 cc. of sample and 9 cc. of distilled water. The alkali used is N/20 NaOH. For none of the media or cultures titrated to date has it been necessary to use more than 3 cc. of the alkali solution.

As a result of numerous experimental titrations conducted at various hydrogen ion concentrations it is found that optimum results are obtained when 8 cc. of formalin are added to the 10 cc. of diluted sample. Lesser amounts of formalin give lower titrations. It is desirable to use no more formalin than necessary because of the high buffer effect of the formalin itself which results in a poor end point if the titration is carried beyond pH 8.0. More than 8 cc. of formalin have been found unnecessary. The effect of using different amounts of formalin is illustrated in table 1.

In the titrations hereafter reported it is to be understood that 8 cc. of formalin were added to 10 cc. of the diluted sample.

TABLE 1
Effect of various amounts of formalin on the formol titration

SAMPLE (1 cc. + 9 cc. H ₂ O)	AMOUNT OF FORMALIN			
	1 cc.	5 cc.	8 cc.	10 cc.
Alanine.....	1.16*	1.94	2.0	2.0
Bouillon.....	0.9	1.03	1.09	1.09

* The figures represent cc. of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank² having been determined for each amount of formalin and subtracted.

There remain to be determined the limits of hydrogen ion concentration between which titration shall be conducted. If one were to titrate an amino-acid directly against sodium hydroxide, i.e., without the use of formalin, he should start the titration at the isoelectric point of the amino-acid and end it at the point of complete neutralization of the amino-acid. In the case of glycine this would be from pH 6.1 to about pH 11.3. However in the case of glycine and other amino-acids the isoelectric point is in a broad isoelectric zone extending in the case of glycine from about pH 5.0 to pH 7.5 or 8.0. Within this zone the degree of dissociation (α) is very slight and even at pH 8.0 is less than 2 per cent.

$$\alpha = \frac{k_a}{k_a + (H^+)} = \frac{1.8 \times 10^{-10}}{1.8 \times 10^{-10} + 1 \times 10^{-8}} = \frac{1.8}{101.8} \text{ at pH 8.0}$$

If a pure monocarboxylic amino-acid is dissolved in distilled water the solution will be found to have a hydrogen ion concentration at or near the isoelectric point, a fact also noted by Eckweiler, Noyes and Falk (1921). If it is titrated with alkali and the titration curve plotted with amounts of alkali added as abscissae and hydrogen ion exponents as ordinates, as was done in the curves to the left in plate 1, the curve will be seen to drop almost vertically towards the alkaline side as the first drops of alkali are added. This represents a portion of the isoelectric zone. Asparagine (plate 2) behaves as a monocarboxylic amino-acid since in this amide one of the carboxylic groups of aspartic acid is in combination with an amide group. Since by the formol titration it is the carboxyl radical and not the amino radical which is titrated it is important that the monoamino-dicarboxylic acids be converted into mono-carboxylic acids, otherwise their titer will be doubled. This may be accomplished by titrating them with sodium hydroxide to the isoelectric point (or to within the isoelectric zone) of their mono-sodium salts. This zone is represented by the nearly vertical portion of the curves in plate 2. To titrate the nitrogen equivalent of the mono-amino-dicarboxylic acids it is therefore necessary to start the titration within the isoelectric zone of their mono-sodium salts and this zone lies within the same region as that of the mono-carboxylic amino-acids.

Within the isoelectric zone a very minute amount of acid or alkali produces a marked change in hydrogen ion concentration. It makes scarcely any difference in titration at what point we start provided it is within the isoelectric zone. The sodium salts of the amino-acids are not titratable after the addition of formalin since they react with formaldehyde to produce not acids but sodium salts of the methylene derivatives of amino-acids. In a solution of glycine at pH 8.4 ($\alpha = \frac{1.8}{41.8}$) about 4.3 per cent of the glycine is present as the sodium salt. If formalin is added at this point the reaction becomes more acid, or rather less basic, because of the formation of methylene derivatives. However there has already been added sufficient alkali to neutralize 4.3

per cent of the methylenglycine and unless this is taken into account, as was done by Sørensen when he dissolved amino-acids in alkali, the formol titration will fall short by a corresponding amount. Before the formalin is added, mixtures of amino-acids, protein digests, bacterial cultures, etc., should be brought to a reaction within the isoelectric zones of all the amino-acids present if this is possible or unless other considerations make it necessary to compromise in regard to this point. We will call this the initial point of the titration. The end point of the titration should be within what may be called the "zone of neutralization" of the amino-acid if it is titrated directly or within the zone of neutralization of the methylene derivative of the amino-acid if formalin has been added. The term "zone of neutralization" will be used to indicate that portion of the titration curve of an acid and base which extends, on either side of the neutral point, nearly vertically to the abscissa with increasing or decreasing hydrogen ion concentrations plotted as ordinates. The neutral point is the point of inflection of the curve within the zone of neutralization and is reached when just sufficient alkali has been added to convert all of the acid into its alkali salt. The neutral point in the titration of the methylene derivative of an amino-acid is at a higher hydrogen ion concentration than that in the titration of the amino-acid itself and it is upon this fact that the usefulness of the formol titration depends. If formalin is added to solutions of various amino-acids and these mixtures are then titrated with sodium hydroxide, the curves plotted on the right in plates 1 and 2 are obtained. In these curves an almost vertical portion is harder to recognize. This is because of the great excess of formalin in the mixtures titrated. Of the 8 cc. of formalin added to each sample a very small fraction of 1 cc. actually reacts with the amino-acid to form the methylene derivative. The remainder exerts a powerful buffer effect on the alkaline side of pH 8.0. Commercial formalin is acid in reaction. It has been our practice to add to it in preparation for each day's work sufficient normal NaOH to reduce the acidity to about pH 5.0, then to titrate 8 cc. of it plus 10 cc. of distilled water to pH 9.0 or beyond with N/20 NaOH, to plot the titration curve, and

use it for the day's experiments. In plates 1, 2, and 3 points determined on these curves are indicated by crosses. If titration values on the formalin curve are subtracted from values at the same hydrogen ion concentration on the amino-acid plus formalin curve the differences may be plotted as a resultant curve lying between the other two. This resultant curve should be the titration curve of the methylene derivative of the amino-acid. Each one of these resultant curves does reach a nearly vertical position which is the zone of neutralization of the methylene derivative and is the end point of the titration. For each of the substances titrated this zone may be said to begin on the acid side at about the hydrogen ion concentration here listed.

	pH
Glycine.....	6.8
Phenylalanine.....	7.6 or 8.0
Asparagine.....	6.0
Glutamic acid.....	8.0
Alanine.....	8.0
Tyrosine.....	7.6 or 8.0
Aspartic acid.....	8.0

A similar zone is found in the resultant curve of the formol titration of ammonium salts (plate 3). In these cases the resultant curve represents the titration of hydrochloric, lactic phosphoric, or carbonic acid. The zones may be said to begin at the following hydrogen ion concentrations.

	pH
Ammonium chloride.....	6.0
Ammonium phosphate.....	8.0
Ammonium lactate.....	6.0
Ammonium carbonate.....	7.0

It will be noted that before the resultant curves reach pH 9.0 their course becomes somewhat irregular or uncertain. This is probably because in the titration of formalin and of formalin plus amino-acids the buffer effect of both substances is so great after they pass beyond pH 8.5 that the end point judged colorimetrically is a very broad and indefinite one. The effect of this is indicated in the cases of glycine, alanine, and asparagine (plates 1 and 2) by shaded regions. The end points of the titrations

might have been taken to lie at any point within these regions. The experimental or technical error is therefore quite large if the titration is carried much beyond pH 8.0 and is in our opinion too large for formol titration by the colorimetric method if carried beyond pH 9.0. The indicators used by us in these regions were phenol red and thymol blue. We found the end point with phenol red at pH 8.0 a very sharp one.¹ At pH 8.0 all of the substances titrated after the addition of formalin had reached the zone of neutralization. This then might be taken as the maximum hydrogen ion concentration at which the formol titration might be completed. An end point of pH 8.2 or 8.4 might be a little better were it not for certain other factors peculiar to the titration of bacteriological media which are discussed below. On the other hand the curves show that pH 8.0 is a little too alkaline to be within the isoelectric zones of some of the amino-acids and ammonium salts. The ideal starting point for formol titration of mixtures of amino-acids would therefore be at a higher hydrogen ion concentration. A hydrogen ion concentration of pH 6.8 as recommended by Henriques and Sørensen (1909) (1910) or of pH 7.0 as employed by Northrop (1921) would serve very well.

However our problem is a special one in that bacteriological media and especially bacterial cultures in such media are more than mere mixtures of amino-acids. They contain among other things volatile and non-volatile fatty acids, phosphates and carbonates, and these substances, especially fatty acids and carbonates together with amino-acids and ammonium salts may change in amount during the growth of the culture. What influence do these substances exert within the range of hydrogen ion concentration chosen for the formol titration? At pH 7.0 the volatile fatty acids are practically neutralized so that they do not enter into a titration with sodium hydrate from pH 7.0 to pH 8.0. The phosphates and carbonates exert large buffer effects between pH 7.0 and pH 8.0, and the amount of alkali

¹ Through the kindness of Dr. van Slyke and Dr. Hastings of the Hospital of the Rockefeller Institute for Medical Research in New York City our color standard at this hydrogen ion concentration was checked electrometrically and found to be correct.

with which they are capable of combining will appear as an error in the formol titration between these limits. If therefore the solution to be titrated is brought to a certain hydrogen ion concentration (e.g., pH 7.0) formalin added and the mixture titrated to a lower hydrogen ion concentration (e.g., pH 8.0 or 8.4) it is absolutely necessary to get rid of the phosphates and carbonates. This may be done by precipitation with barium and filtration.

The following method has given the most accurate results with bacteriological media and with mixtures of amino-acids, phosphates and carbonates.

Method A. Measure out accurately with an Ostwald pipette 2 cc. of culture medium. Add from a burette exactly 2 cc. of $N/5$ or stronger NaOH. Add a small piece (about 0.2 gram) of barium chloride. Shake to dissolve the barium chloride and allow to stand for a few minutes. Pour onto a small dry paper filter.

With the Ostwald pipette measure out 2 cc. of the filtrate into a large test tube (1 inch in diameter) such as can be used in the comparator block. Caution: Do not blow through the pipette used for measuring the sample since the CO_2 from the breath will cause the fluid to become cloudy.

Add 5 drops of phenol red solution and sufficient $N/5$ HCl to bring the reaction of the sample near pH 7.0 and then sufficient distilled water to bring the contents of the tube to about 10 cc. which may be judged by a mark on the side of the tube. Bring the reaction of the sample to pH 7.0 with $N/20$ HCl or NaOH, using the comparator block with 1 cc. of medium plus 9 cc. of water as a color screen behind the colorimetric standard.

Add 4 drops more of the phenol red solution and 8 cc. of formalin.² Titrate with $N/20$ NaOH to pH 8.0 or 8.4. From this result subtract

² It is our experience that if formalin is made alkaline it does not "keep" well, i.e., the hydrogen ion concentration increases slowly but appreciably within two hours. If, however, it is made slightly acid it does not change appreciably during the working day. Instead of adjusting the reaction of the formalin to pH 8.0 or 8.4 before adding it to the sample of medium we prefer to prepare it as follows: To a sufficient quantity for the days' work is added normal NaOH (usually less than 1 cc. per 100 cc. of formalin) until the reaction is between pH 5.0 and pH 7.0. The formalin blank consists of 8 cc. of this formalin plus 10 cc. of distilled water titrated against $N/20$ NaOH to pH 8.0 or 8.4 (the end point chosen for the formol titrations).

the titration of the formalin blank.² The remainder multiplied by 5 is the formol titration expressed in terms of per cent normal, *i.e.*, the percentage normality of substances reacting with formaldehyde to produce titratable acids.

In the formol titration of bouillon we have obtained exactly the same values when titrating to pH 8.0 as at pH 8.4 whereas the colorimetric end point at pH 8.0 is somewhat sharper than at pH 8.4 because of the greater buffer effect of the formalin at pH 8.4.

A briefer method than the above, one which requires less material and which gives almost identical results even in the presence of large amounts of carbonates and phosphates in bouillon, is as follows:

Method B. With an Ostwald pipette measure out 1 cc. of the medium or culture into each of two large test-tubes (1 inch in diameter) such as can be used in the comparator block. To each tube add 9 cc. of ammonia-free distilled water or better sufficient water to bring the contents of both tubes to the same level. One of the tubes serves as a color screen in the comparator block. To the other tube, hereafter referred to as the sample, add 5 drops of phenol red (the same proportion of indicator as is contained in the colorimetric hydrogen ion standards).

Bring the reaction of the sample to pH 8.0 by the cautious addition of $N/20$ NaOH or HCl³ as needed. A few drops are usually sufficient.

To the sample add 4 drops more of phenol red and then to both the sample and the color screen tubes add 8 cc. of formalin.² Twirl the sample tube just sufficiently to mix the formalin with the sample and as quickly as possible but with as little agitation as possible, add from a burette³ $N/20$ NaOH until the pink color of the indicator is visible, then more deliberately titrate to pH 8.0. After subtracting the titration of the formalin blank the result multiplied by 5 is the formol titration expressed in terms of per cent normal. By multiplying the latter result by 14 the result may be expressed as milligrams of nitrogen per 100 cc. of medium.

³ The burette used should be finely graduated so that it can be read in hundredths of a cubic centimeter. The $N/20$ NaOH and HCl used should contain phenol red in the same concentration as is present in the sample and in the colorimetric standards, a suggestion adopted from Hurowitz, Meyer, and Ostenberg (1915).

The method above described resembles that of Kendall, Day and Walker (1913) and that of Berman and Rettger (1918) in that the sample is brought to a certain hydrogen ion concentration before formalin is added and titrated back to the same hydrogen ion concentration after the addition of the formalin, but it differs from them in other respects. Kendall, Day and Walker allowed the sample of medium to stand thirty minutes after the addition of formalin before proceeding with the titration. We have found this not only unnecessary but distinctly bad because of the loss of CO_2 into the atmosphere. The CO_2 may be removed before the formalin is added in which case any ammonium carbonate which was present is transformed into other ammonium salts and there will be no further loss of CO_2 after the formalin is added, but the escape of CO_2 after the formalin is added means a decreasing titratable acidity and hence lowers the formol titration. The CO_2 must either be removed before adding the formalin or must be kept in solution until the titration is finished. If in the presence of carbonates it is desired to allow the mixture of formalin and sample to stand for a few minutes it should first be brought to an alkaline reaction. The effect of the escape of CO_2 into the air is illustrated by the formol titration curve of ammonium carbonate in plate 3. This titration was carried out with four different indicators in four different samples of ammonium carbonate solution. Formalin was added to the first sample (methyl red) and it was titrated deliberately from pH 5.2 to 5.6. Formalin was added to a second sample (brom cresol purple) and it was titrated from pH 6.0 to 6.8. Similarly a third sample (phenol red) was titrated from pH 7.0 to 8.4. The fourth sample (thymol blue) was titrated from pH 8.8 to 9.0. It will be noted that as the titration of each sample progressed the curves ending on the acid side of pH 7.0 fell short, so as to produce breaks or steps in the entire curve at A and B, a condition which did not occur in the absence of carbonates, as is illustrated by the curves of the other substances similarly titrated. We attribute these breaks to loss of CO_2 . This is further illustrated in table 2.

TABLE 2

Illustrating the effect of loss of CO₂ during the formal titration

SAMPLE	PROCEDURE	RESULT
Glycine.....	Titrated deliberately	2.53*
Glycine + 1 per cent NaHCO ₃	Titrated several minutes after addition of formalin	2.34
Glycine + 1 per cent NaHCO ₃	Titrated quickly after addition of formalin	2.52
Glycine + 1 per cent NaHCO ₃	Titrated under oil	2.55
Glycine + 1 per cent NaH ₂ PO ₄	Titrated deliberately	2.54

* In this and the succeeding tables the results are expressed as cubic centimeters of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank² having been subtracted.

TABLE 3

Formol titrations in the presence of carbonates and phosphates

SAMPLE	INITIAL POINT OF TITRATION	END POINT OF TITRATION	RESULT
	pH	pH	
Bouillon only (containing only the phosphates and carbonates which are native to meat infusion bouillon)	7.0	8.0	1.35
	7.0	8.4	1.35
	8.0	8.0	1.07
	8.4	8.4	0.96
Bouillon + 1 per cent NaHCO ₃ and 1 per cent Na ₂ HPO ₄ (titrated quickly)	7.0	8.0	1.9
	8.0	8.0	1.06
Bouillon + 1 per cent NaHCO ₃ and 1 per cent Na ₂ HPO ₄ (carbonates and phosphates precipitated by BaCl ₂ and filtered out)	7.0	8.0	1.13
Glycine only	7.0	8.0	1.24
	8.0	8.0	1.22
Glycine + 1 per cent NaHCO ₃ and 1 per cent Na ₂ HPO ₄ (titrated quickly)	8.0	8.0	1.22
Glycine + 1 per cent NaHCO ₃ and 1 per cent Na ₂ HPO ₄ (carbonates and phosphates precipitated by BaCl ₂ and filtered out)	7.0	8.0	1.26

By bringing the reaction back to alkaline as quickly as possible after the addition of formalin the loss of CO_2 into the air is minimized. By adjusting the reaction of the sample to pH 8.0 and carrying the final titration to the same point the buffer effect of phosphates, carbonates, or other buffer substances in the medium is eliminated. That by these means the necessity for removing the phosphates and carbonates by precipitation is obviated is illustrated in tables 2 and 3. Other means have been tried, such as preliminary acidification and boiling or aeration to get rid of the CO_2 , or titration under a layer of oil, but the technic described above is almost if not quite as efficient and much simpler.

From a study of the titration curves of pure amino-acids and ammonium salts already described it was concluded that optimum results were obtained by adjusting the reaction of the sample to pH 6.8 or 7.0 then adding the formalin and titrating to pH 8.0 or 8.4. However, with bouillon, and particularly if phosphates or carbonates have been added, titration between these limits of hydrogen ion concentration is impossible as is also illustrated in table 3. To eliminate the buffer effect of these substances the formol titration must start and end at the same hydrogen ion concentration. It is therefore imperative to determine what this hydrogen ion concentration should be. For each of the pure substances titrated formol titrations were made between such limits as pH 5.6 to 5.6, pH 6.8 to 6.8, pH 7.0 to 7.0, pH 7.6 to 7.6, pH 8.0 to 8.0, pH 8.4 to 8.4, and pH 9.0 to 9.0. These titrations are indicated by small triangles in plates 1, 2 and 3.⁴ The results are in strict agreement with what might be deduced from the character of the other curves plotted. If the isoelectric zone of the amino-acid overlaps a portion of the zone of neutralization of the methylene derivative (resultant curve) (i.e., if the two curves fall nearly vertically at

⁴ For all of the solutions, the titrations of which are plotted in plates 1, 2 and 3, total nitrogen or ammonia determinations were made. On the assumption that these determinations represent 100 per cent of the substance in solution the curves are so adjusted that a perfect formol titration (i.e., titration of 100 per cent of the substance) should require 2 cc. of $\text{N}/20\text{NaOH}$. A solution of this concentration would be one-tenth normal.

the same hydrogen ion concentration) the formol titration gives practically perfect results when carried out between limits of hydrogen ion concentration within the overlapping portion of these zones. For example the isoelectric zone of glycine (plate 1) and the zone of neutralization of its methylene derivative (resultant curve) overlap from about pH 6.8 to 8.0 and practically 100 per cent of the glycine may be determined by formol titration from pH 6.8 to 6.8 or from pH 8.0 to 8.0. However on either side of these extremes, as shown by the triangles at pH 5.6, 8.4 and 9.4, lower values are obtained. In the case of alanine there is only slight overlapping of the zones at pH 8.0 to perhaps 8.4. In the case of asparagine, ammonium chloride and ammonium lactate there is overlapping from about pH 6.0 to 7.0. In the case of the other amino-acids titrated there is no actual overlapping of the zones, consequently with these substances all formol titrations which begin and end at the same hydrogen ion concentration are too low, and if we are to use the method under consideration the hydrogen ion concentration at which a maximum titration is obtained must be found. Maximum titration values for certain amino-acids were obtained at the following hydrogen ion concentrations; phenylalanine, pH 7.6; tyrosine, pH 8.0; aspartic acid, pH 8.4; glutamic acid, pH 8.0 to 8.4. We did not have the difficulty experienced by Sørensen (1907) (1908) of obtaining results that were too high in the formol titration of tyrosine. With ammonium phosphate and ammonium carbonate another phenomenon is encountered; the line of the formol titrations starting and ending at the same hydrogen ion concentration crosses the titration curves of phosphoric and carbonic acids (resultant curves). The formol titration of ammonium phosphate at points to the acid side of pH 7.8 leads to results which are too high while titration at points to the alkaline side of pH 8.0 gives low results. Perfect results are obtained at pH 7.8 or 8.0. Formol titration of ammonium carbonate did not yield 100 per cent of the ammonia value probably because of the escape of some of the CO_2 during titration, but maximum results (at least 90 per cent of the theoretical) were obtained at pH 6.0, 7.0, or 8.0.

If it is desired to perform a formol titration of a mixture of amino-acids and ammonium salts from a selected hydrogen ion concentration back to the same hydrogen ion concentration, as here proposed, the optimum hydrogen ion concentration will evidently depend upon the relative proportions of the various amino-acids and ammonium salts present. This optimum hydrogen ion concentration has been determined empirically for solutions of "aminoids," peptone and standard meat infusion bouillon (see table 4).

TABLE 4

Formol titrations of bacteriological media at various hydrogen ion concentrations

MEDIUM	THE INITIAL AND END POINTS OF TITRATION				
	pH 7.2-7.2	pH 7.6-7.6	pH 8.0-8.0	pH 8.4-8.4	pH 9.0-9.0
1 per cent beef aminoids*.....	0.86	0.9	0.95	0.79	0.64
1 per cent casein aminoids*.....	0.68	0.71	0.74	0.65	0.36
1 per cent Fairchild peptone.....	0.68	0.67	0.68	0.52	0.39
	(pH 7.0-7.0)				
Veal bouillon 1502.....	1.04	1.12	1.11	1.04	
Veal bouillon 1502 + 1 per cent NaH ₂ PO ₄	1.35	1.23	1.10	1.04	
Veal bouillon 1502 + 1 per cent NaHCO ₃	0.81	1.04	1.07	0.99	

* Arlington Chemical Co. product.

The "aminoids" gave maximum formol titrations at pH 8.0. Fairchild peptone gave maximum results between pH 7.2 and pH 8.0. The veal infusion bouillon gave maximum titrations at pH 7.6 and pH 8.0. In the case of other samples of bouillon we have found the maximum more often at pH 8.0 than at pH 7.6. When NaHCO₃ or NaH₂PO₄ was added to the bouillon practically the same result was obtained at pH 8.0, but in the phosphate bouillon higher titrations were obtained at pH 7.6 and at pH 7.0. This phenomenon is related to a similar peculiarity of the ammonium phosphate curve (plate 3) already commented upon. This fact and the comparison with the titrations of the plain bouillon and other substances serve to indicate that the formol titration of bouillon from pH 8.0 to 8.0 gives the most

nearly correct value, a value very close to that which may be obtained after removal of the phosphates and carbonates. In the case of pure amino-acids and ammonium salts the formol titration from pH 8.0 to 8.0 yields from 90 to 100 per cent of the theoretical value, depending upon the individual substance titrated.⁵

It will be of interest to know how the results so obtained compare with those obtained by the methods of Kendall, Day and Walker (1913), Foster and Randall (1921), Kendall (1922),

TABLE 5

Comparison of the results of formol titration by the methods of Kendall, Berman and Rettger, and the method here described

SAMPLE	METHODS OF		
	Kendall	Berman and Rettger	The author
Glycine.....	2.27*		2.58
Asparagine.....	0.86		1.11
Veal bouillon.....	0.86	0.65	1.07

* For purpose of comparison all the results are reduced to the same terms. The figures represent cc. of N/20 NaOH required for the titration of each 1 cc. of sample, the formalin blank* having been subtracted in the author's method, the formalin having been "neutralized" to phenolphthalein in the methods of Kendall and of Berman and Rettger.

and Berman and Rettger (1918). The results recorded in table 5 serve to show that the results of Kendall's method are probably at least 20 per cent too low and those of Berman and Rettger even lower. Assuming that the ammonia determinations in the work of these authors were correct it will be seen that when the ammonia nitrogen was subtracted from the formol nitrogen the resulting amino nitrogen value probably involved an error even greater than that indicated by the formol titration alone. Three factors must have conspired to produce these

⁵ Eckweiler, Noyes and Falk (1921) have published a titration curve of glycylglycine which is said to be similar to that of other simple dipeptides. It is to be noted that according to this curve the formol titration of these substances from pH 8.0 to 8.0 would introduce a larger error than that found in the titration of simple amino-acids. However, in the case of standard meat infusion bouillon at least, the sum of errors is not a large one.

errors: (1) the use of too much water and too little formalin, (2) the titration from pH 8.3 or 8.4 (the end point of phenolphthalein) back to pH 8.3 or 8.4, and (3) the loss of CO₂ from the sample after formalin had been added.

In criticism of the method of Sørensen it was claimed by de Jager (1909) that a mixture of glycine and ammonium salt gave a formol titration which was lower than the sum of the formol titrations of the glycine and ammonium salt made separately. Henriques and Sørensen (1910) found this of little consequence

TABLE 6
Formol titration of mixtures of an amino-acid and an ammonium salt

SAMPLE	FORMOL TITRATION (pH 8.0 to 8.0)
1 cc. glycine solution.....	2.53
1 cc. ammonium lactate solution.....	1.37
Sum.....	3.90
Mixture of 1 cc. glycine solution and 1 cc. ammonium lactate solution.....	3.91

TABLE 7
Formol titration of an amino-acid added to bouillon

SAMPLE	FORMOL TITRATION (pH 8.0 to 8.0)
1 cc. glycine solution.....	1.17
1 cc. bouillon.....	1.09
Sum.....	2.26
Mixture of 1 cc. glycine solution and 1 cc. bouillon.....	2.26

in the titration of urines. In table 6 it is shown that this is not a disturbing factor in the method here described.

In the growth of bacterial cultures amino-acids are often liberated from protein substances in the medium. It is therefore of direct interest to know whether the amount of such acid liberated may be actually measured by the formol titration. The formol titration of bouillon before and after the addition of a titrated amount of glycine indicates that it can be so measured (see table 7).

SUMMARY

The influence of certain factors involved in the formol titration of amino-acids and ammonium salts is illustrated. Their titration curves are plotted.

Serious errors in current methods of performing the formol titration of bacteriological media are pointed out.

It is shown that the formol titration of bacteriological media presents a special problem. Simple methods of performing this titration are described, methods requiring very small samples of media. Method A is a modification of the technique of Henriques and Sørensen for the formol titration of urine and involves the removal of carbonates and phosphates by precipitation with barium. Method B is almost as accurate as A, yielding results which probably represent 94 per cent of the amino-acids and ammonia present, and does not require the removal of carbonates and phosphates.

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EXPLANATION OF PLATES

1. —○— titration curve of 1 cc. of sample plus 9 cc. water.
2. —×— titration curve of 8 cc. formalin plus 10 cc. water.
3. —⊕— titration curve of 1 cc. sample plus 9 cc. water and 8 cc. formalin.
4. —●— resultant curve obtained by subtracting values on curve 2 from those on curve 3.
5. Δ formol titrations.

Note: The substance titrated, i.e., the sample, is indicated by the name printed beneath each set of curves.

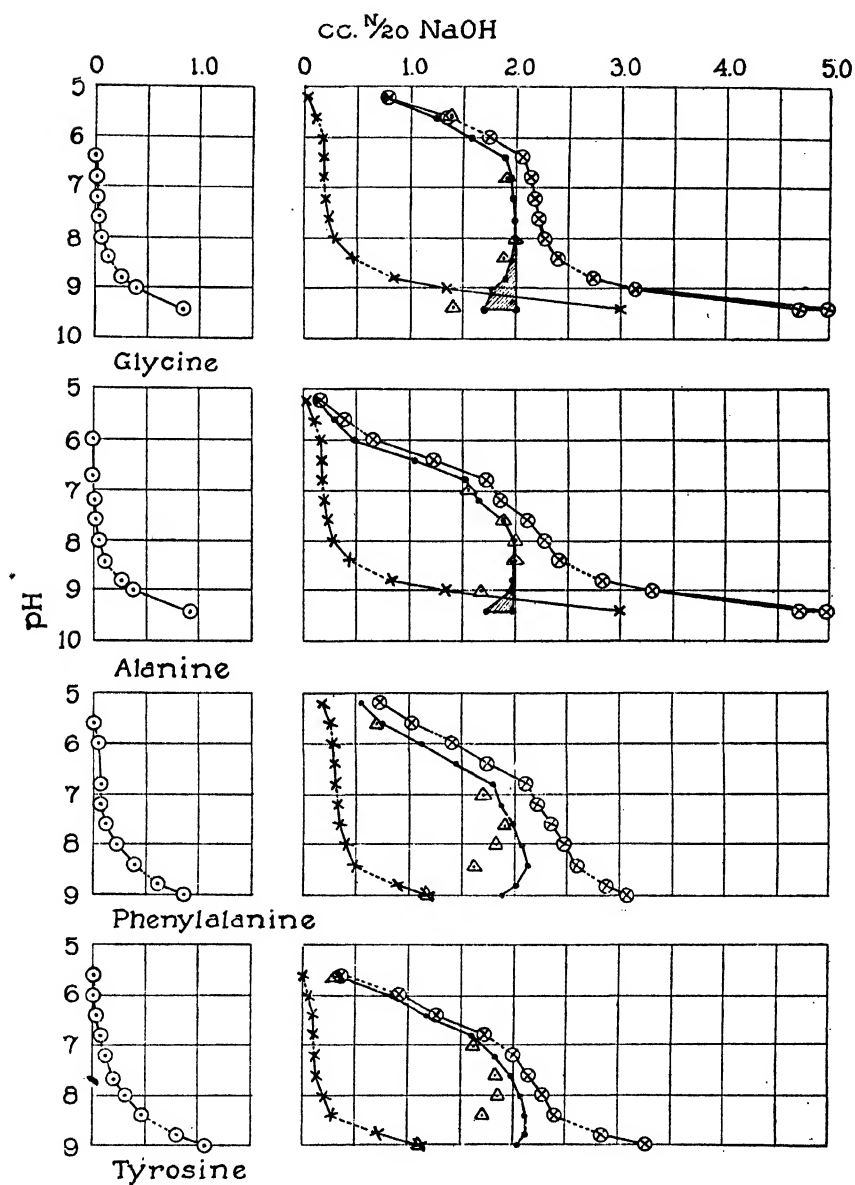


PLATE 1

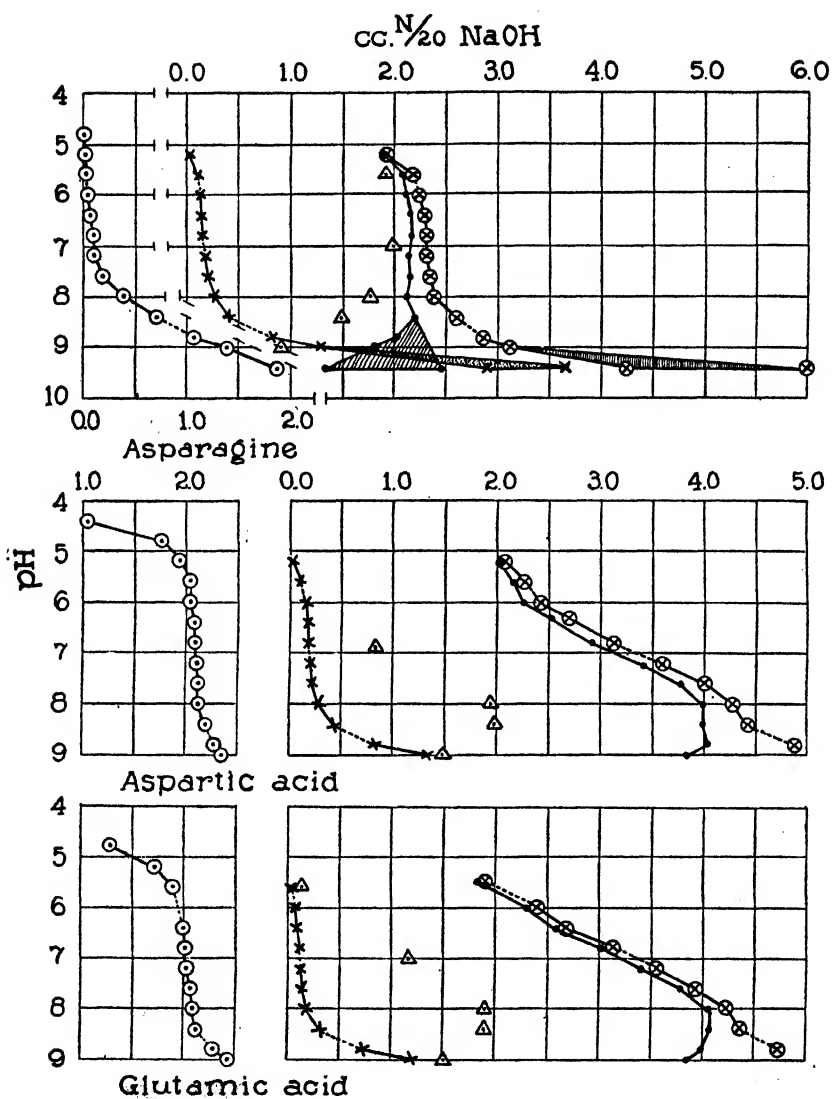


PLATE 2

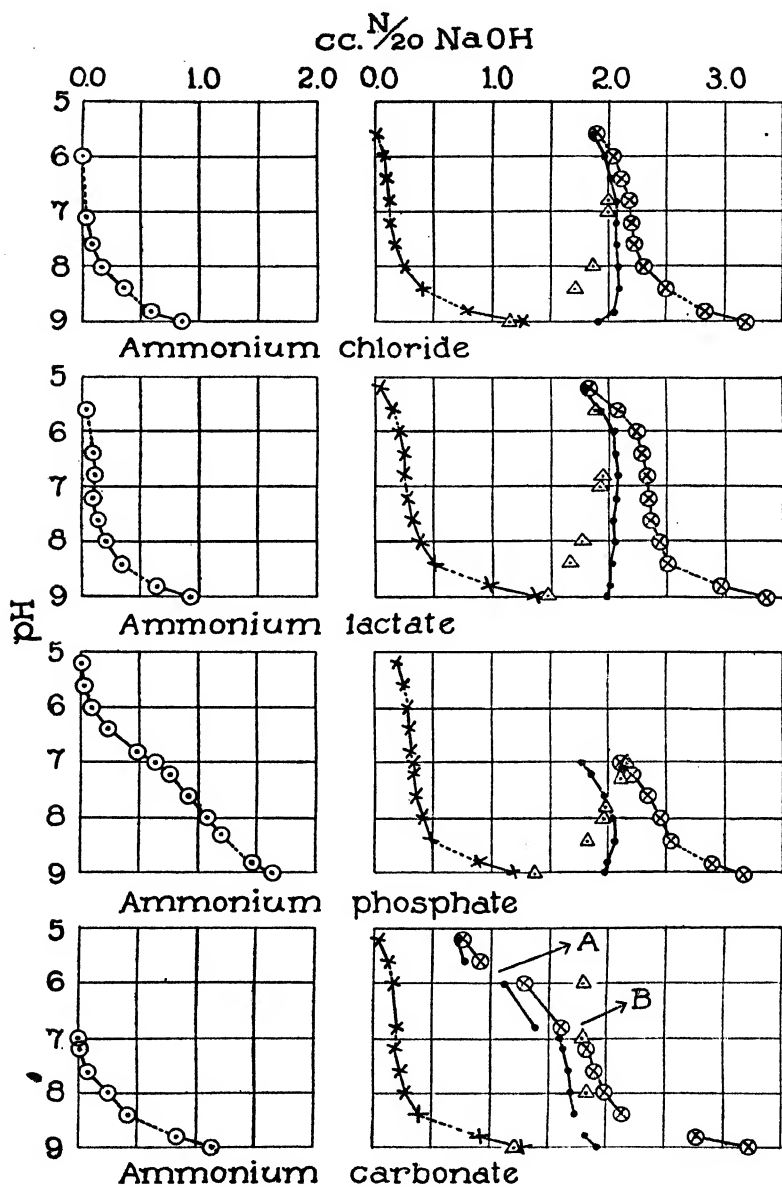


PLATE 3

CLOSTRIDIUM BOTULINUM

IV. RESISTANCE OF SPORES TO MOIST HEAT

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The several recent outbreaks of botulism, which have been traced to foods preserved by some method of heating, have focused critical attention on the thermal resistance of the spores of *Clostridium botulinum*. While several papers have been published on this subject, its importance justifies repetition of the work in order that the conclusions based on the data, may be as accurate as possible. The present investigation was initiated before some of the other papers were published and was continued even though the experiments repeated some of those already reported.

HISTORICAL

Two papers have been published by Weiss (1921a, 1921b) which bear directly on the subject under discussion. He reported that the free spores of *Clostridium botulinum* were destroyed within five hours at 100°C., within forty minutes at 105°C., and within six minutes at 120°C. Weiss believed that the spores were injured before death and that the death process was a gradual one. Such an opinion is in keeping with our knowledge of disinfection by moist heat. Young spores were more resistant than old ones. Those one month old were found to be many times more resistant than older ones. The hydrogen ion concentration was found to have the usual effect. In the second paper Weiss reported some data on the thermal resistance of spores in canned food liquors. The resistance, as would be expected, varied with the reaction of the food. In gooseberry juice the spores were killed in 30 minutes while about

180 minutes were required for killing the spores in lima bean juice. Burke (1919 a) reported that the spores could survive boiling for three hours or more. In another paper Burke (1919 b) reported that the spores grown in brain medium were more resistant than those grown in broth. Fifteen pounds pressure for ten minutes was believed to be insufficient for destroying the spores. Thom et al (1919) reported that the Boise strain withstood a pressure of 10 pounds for fifteen minutes or 100°C., for one hour. Fifteen pounds for fifteen minutes in the autoclave destroyed it.

EXPERIMENTAL

Spore suspensions and cultures. The cultures used in these determinations were obtained from Dr. Robert Graham, Department of Animal Pathology, University of Illinois. They were numbered as follows with source: 820-A, isolated from garbage which caused avian botulism in Urbana, type A organism; 854-A, also type A, obtained from the spleen of a calf which died showing all of the characteristic symptoms of botulism; 820-B, a type B organism, isolated from corn ensilage which was known to have caused botulism among cattle in Iroquois County; G-6-B, another type B strain, isolated from oat hay, which, when inoculated into monkeys, produced death with the characteristic symptoms. In producing the spores of *Clostridium botulinum* a brain medium of thinner consistency than that described by Weiss (1921a) was utilized.

Medium. This was made by adding one part of finely macerated sheep's brain to two parts of water. It was then sterilized in an autoclave at 15 pounds pressure for thirty minutes in 250 cc. Pyrex Erlenmeyer flasks, from 125 to 150 cc. of medium in each flask. This medium was used without adjustment to grow the organism and produce the spore suspension since it was found to be practically neutral (pH 6.8 to 7.0). Dickson and Byrke reported the use of a medium consisting of a 1 per cent glucose peptic digest of liver broth adjusted to pH 7.3 to 7.5.

Inoculations. Inoculations were made by means of 10 cc. sterile pipettes, 10 cc. of a stock suspension in brain medium

being transferred to each flask. Melted paraffin was then poured into the flask which hardened to give a coating on top of about 1 cm. in thickness. At a later date we tried growing the organisms without covering the culture with paraffin to determine whether a larger number of spores could be produced in the suspension, but have not sufficient data at present to warrant any conclusions. This procedure has been suggested by various investigators for certain of the other pathogenic anaerobes.

The flasks were then incubated at room temperature for approximately 30 days in the dark. To determine the number of spores produced the Torrey anaerobic plate was used. The medium used was a 0.5 per cent glucose agar in which dilutions of 1 to 1000 and 1 to 10,000 were inoculated. A covering of glucose agar was then poured on top and finally a coating of paraffin, colored with Sudan III, as suggested by Northrup to make the colonies stand out in relief. These cultures were incubated from ten days to two weeks and counted. The average number of spores obtained from the dilution plate counts was 3,200,000 per cubic centimeter.

Apparatus. Thermal determinations were made using a DeKotinsky electric bath, with a thermo regulator attachment, turbine agitator, Nujol for the liquid of high boiling point, and Wasserman test tube rack for immersion of tubes. Temperatures of 100°C.; 105°C.; 110°C.; 115°C.; and 120°C.; were used in the experimental heating of the organisms.

The cultures used were transferred by means of a sterile pipette from the 250 cc. flasks to sterile, special soft glass tubes (as recommended by Bigelow and Estey (1920), having an inside bore of 7 mm., an outside diameter of 9 mm., and 250 mm. in length), 1 cc. of media to each tube. The tubes were then drawn out, 70 mm. above the top of the medium, to obtain a short capillary neck above the medium. One set was then exhausted to give a vacuum of about 17 mm. and sealed at the capillary portion. The second set was not exhausted but left plugged with cotton and heated as open tubes.

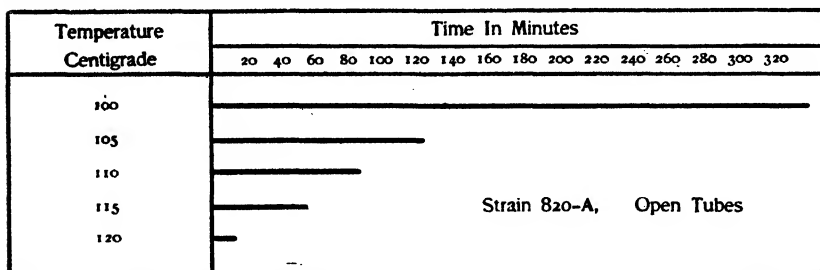
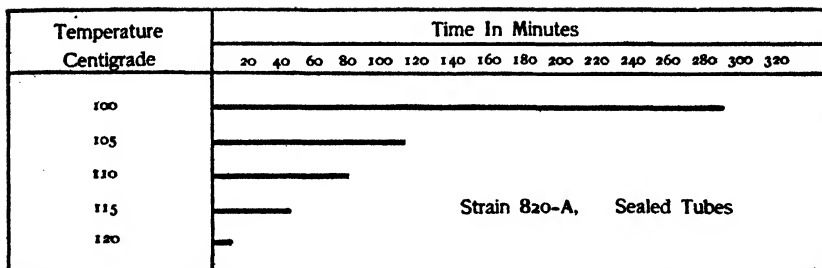
It was found to be troublesome to use the open tubes at temperatures above 100°C., since the medium boiled up in the tubes to the plugs and gave opportunity for too great an error. To

overcome this we drew out the tube in the same manner as for the sealed tubes, and pushed a tight cotton plug down until it was stopped by the capillary constriction. After heating, and immediately as each tube was taken from the bath, it was sealed in a flame to prevent any material which may have been unevenly heated from being drawn down into the tube as it cooled.

The spores used in these tubes were about thirty days old, it being impractical to utilize those exactly thirty days old at all times. The variation in resistances of a few days when the spores were approximately thirty days old was shown by Weiss to be negligible.

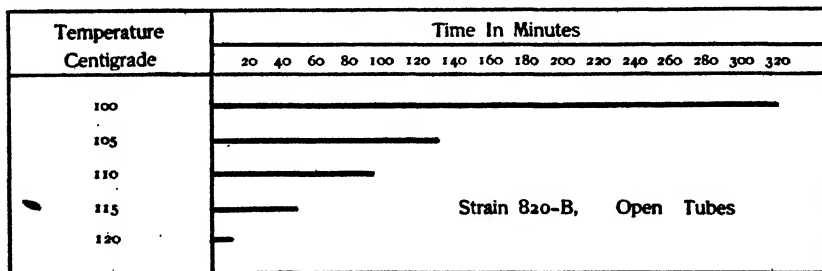
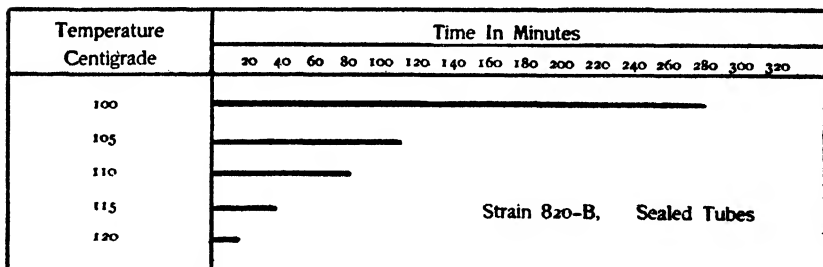
Incubation. One tube of each culture was taken out of the oil bath at stated intervals, cooled at once by immersion in cold water, and inoculated into 10 cc. tubes of sterile brain medium, care being taken to mix the spore suspensions with the brain medium. As stated above, the open tubes were sealed immediately and then cooled. The tubes of brain medium were then covered with paraffin to a depth of about 1.5 cm., plugged and incubated at room temperature in the dark. Owing to the time of completion of the thermal determinations, these tubes were incubated over the summer vacation and averaged an incubation period of four months.

Inoculation. All cultures showing gas formation with the characteristic foul odor were discarded as indicative of active growth and the first one of the series showing no gas formation was taken for further study to ascertain if there had been any growth and toxin formation. One cubic centimeter of this latter culture was fed to a guinea pig through a sterile pipette, and the pig kept under observation for one month. If death with the characteristic symptoms did not ensue in this time the spores were considered to have been destroyed. It was found that our results supported the observations of Weiss, that the spores were progressively injured before death finally ensued. Some pigs inoculated orally, lived for periods of from three to nineteen days, showing that the organisms had been so injured that only a small amount of toxin or perhaps a toxin of low virulence had been produced.



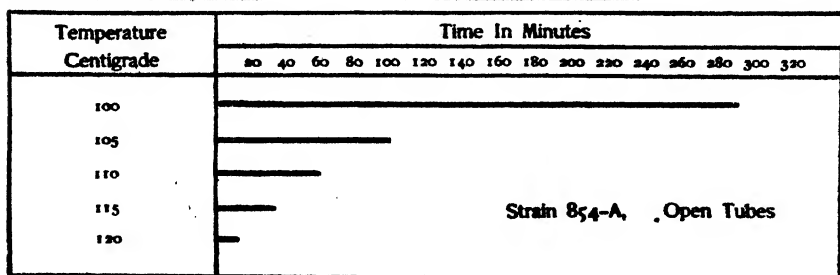
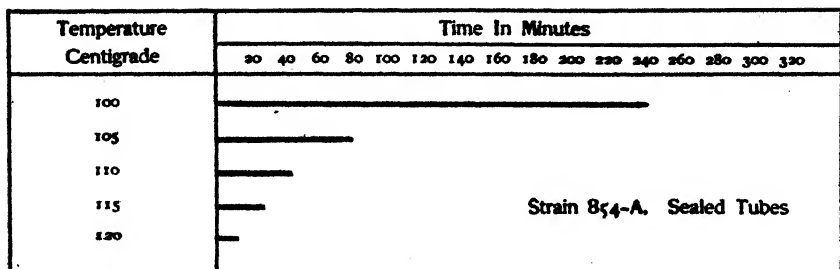
STRAIN 820-A

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.



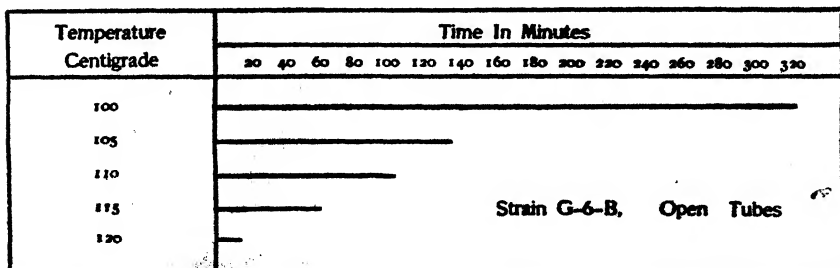
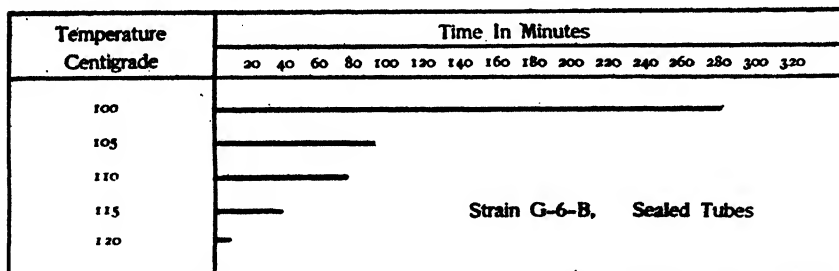
STRAIN 820-B

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.



STRAIN 854-A

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.



STRAIN G-6-B

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.

DISCUSSION OF RESULTS

From the accompanying tables it will be seen that there is a variation in resistance of different strains of spores under controlled conditions. The most resistant spores were found to be destroyed in less than five hours at 100°C. in the cultures which had been exhausted to obtain a 17 mm. vacuum, and in five and one-half hours in the open tubes. Bigelow and Estey (1920) stated that the time necessary for the tubes to reach 100°C, after being immersed in the bath was on the average 15 seconds since the tubes used were small in diameter, only moderately thick, and completely immersed in the oil bath.

At 105° the cultures showed a variation of thirty minutes in death points obtained, the most resistant being destroyed in one hour and fifty minutes for the tubes exhausted to 17 mm. vacuum. In the open tubes there was also a variation of 30 minutes, the most resistant cultures being killed in two hours and ten minutes. The same conditions hold for 110°C., the cultures varying ten minutes and showing an irregularity in the final death point as determined by injection into guinea pigs. All cultures were found to be destroyed in less than an hour and a half in the sealed tubes. The open tubes showed a greater variation in thermal death time, the most resistant being killed in one hour and forty minutes, while the least resistant one was destroyed in fifty-five minutes. At 115°C. we see less variation in death point in vacuum tubes, all cultures being killed in forty minutes in the sealed tubes. The open tubes required a slightly longer time, the most resistant spores being destroyed in fifty minutes. At 120°C. no cultures survived ten minutes heating but 820-A does show slight toxin formation at the end of five minutes heating. This tube when inoculated into brain medium, incubated three months at 21°C., and fed to a guinea-pig, required nineteen days to cause death. All other cultures caused death of the guinea-pigs in twelve to seventy-two hours.

The variation between the open tubes and those exhausted and sealed to give a vacuum of 17 mm. is small but the time required in the case of the open tubes was always found to be longer.

The change in pH during the process of heating was found to be so small as to be negligible, the tubes having first been soaked over night in cleaning solution. The change in four hours with the tubes not so cleaned was found to average 0.6 by Morrison (unpublished data).

The reaction of our media was found by numerous determinations to average pH 6.8. This was kept in Pyrex flasks and used without adjustment. It was found that brain medium put up in soft glass flasks and kept for periods of twenty-one days or more had an average reaction of about pH 7.4. Either medium could be used to grow our cultures, both apparently being of equal value. With media testing pH 6.8, growth of the organism produced a gradual change to the alkaline side, reaching pH 7.2; with media at pH 7.4, instead of producing greater alkalinity, it, too, reached an average of 7.2. Weiss reports the pH as becoming stable at 7.5 after cultural growth in brain medium.

SUMMARY AND CONCLUSIONS

1. The spores of *Clostridium botulinum* in sealed tubes exhausted to 17 mm. vacuum are destroyed within a period of five hours at 100°C.; within two hours at 105°C.; within one and one-half hours at 110°C.; within forty minutes at 115°C.; and within ten minutes at 120°C.

2. A longer heating period was required to destroy spores of the same age in open tubes than in tubes exhausted and sealed, under the conditions that obtained in this investigation.

3. The death of spores is probably a gradual process. Surviving spores may have their internal mechanism so injured that weak toxins are formed.

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THE FERMENTATION OF ARABINOSE AND XYLOSE BY CERTAIN AEROBIC BACTERIA¹

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During a study of the aerobic flora from lake water, silage and vinegar, it was found that many of these organisms attack pentose sugars. In contrast to the acid formers which are described in earlier papers (Fred et al, 1919; 1921), these aerobic organisms ferment the pentose sugars, arabinose and xylose more slowly and produce only a small amount of acid. The total yield of fixed acid is far too small to account for the carbohydrate consumed. Products other than acids must be formed and various experiments were carried out in order to study the products of the aerobic fermentation and the proportion in which they are found.

Six cultures of bacteria representing various groups of the more common aerobic types were used in this study. *Bacillus vulgatus* (Flügge) Trevisan, synonymy *Bacillus mesentericus vulgatus*, was isolated from the mud of Lake Mendota; *Acetobacter sorbose* and *Acetobacter xylinum* were obtained from the American Museum of Natural History, and originally came from Bertrand; while three chromogenic forms, one a rod which answers to the description of *Bacterium herbicola aureum* (Düggeli 1904) and the others two coccus forms designated "Organism A, and B" were isolated from green sweet corn.

Some of the characteristics of these aerobic pentose fermenters are shown in table 1. Aside from the great difference in morphology, these forms are readily distinguishable by definite

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TABLE 1
The characteristics of certain aerobic pentose fermenters

NUMBER	BACILLUS VULGATUS	ACETOBACTER SORBOSE	ACETOBACTER XYLINUM	ORGANISM A	ORGANISM B	BACTERIUM HERBICOLA AUREUM
1. Form.....	Rod	Rod	Rod	Coccus	Coccus	Rod
2. Spores.....	Present	None	None	None	None	None
3. Flagella.....	Present	Present	Present	None	None	Present
4. Pigment.....	Fawn-colored	Light-yellow	Brownish-yellow	Orange	Yellow	Light-yellow
5. Gram stain.....	Positive	Positive	Positive	Positive	Positive	Negative
6. Catalase test.....	Very strong	Strong	Fair	Strong	Very strong	Very strong
7. Carbinol test						
Glucose.....	Strong	Fair	Medium	None	None	None
Arabinose.....	Strong	Trace	Medium			
Xylose.....	None	None	None			
8. Gelatin.....	Liquefied	Not liquefied	Not liquefied	Slow liquefaction	Rapid liquefaction	Not liquefied
9. Litmus milk.....	Reduced, soft curd later digestion	Reduced, brown serum zone, soft curd	Reduced, pink serum zone, soft curd	Alkaline, reduced	Alkaline, digestion	Yellow, no change

physiological characters. These differences are perhaps best seen from a comparison of the action of the bacteria in milk and in gelatin. On solid and liquid media, *B. vulgatus* forms a wrinkled membrane which spreads rapidly over the entire surface. *Acetobacter sorbose* on glucose yeast-water agar forms a pale yellow film. In liquid the medium becomes turbid throughout. *Acetobacter xylinum* forms a thick pink to brown membrane on glucose yeast-water agar slopes. The coccus forms A and B produce a raised profuse growth on agar slopes and a decided turbidity in liquid media.

In yeast water solutions the three chromogenic forms ferment glucose, mannose, fructose, sucrose, maltose, lactose, melezitose salicin, mannitol and glycerol with the production of a small amount of fixed acid. Raffinose, alpha-methyl glucoside, dulcitol, inulin and starch are not attacked. Aside from total acidity, the amount of sugar fermented was also determined. In general it was found that *B. herbicola aureum* is the most active fermenter of the three, destroying from 50 to 60 per cent of the sugar in the first ten days. Organism B in turn is more active in the fermentation of sugars than Organism A.

THE FERMENTATION OF ARABINOSE AND XYLOSE BY *B. VULGATUS* *ACETOBACTER SORBOSE* AND *ACETOBACTER XYLINUM*

In preliminary experiments to determine the fermentation of the pentoses by these organisms 25 cc. cultures of 2 per cent sugar medium were grown in 100 cc. Erlenmeyer flasks. The source of the nitrogen was varied. However, no well defined difference in fermentation was noted from the use of beef broth or of peptone broth in place of the yeast water. In general, the yeast water proved slightly superior and hence in all of the quantitative work which follows, this medium was employed.

The methods for measuring the products of fermentation with these aerobic forms were essentially different from those used in the work on acid fermentation of pentoses by *Lactobacilli*. The fermentation was carried out in a closed system, figure 1, through which carbon dioxide-free air was drawn. The fermentation flask C was closed with a two-hole rubber stopper, which

fitted tightly in the neck of the flask (see *F*). Through one hole was passed a long glass tube reaching almost to the surface of the culture solution. The other end of this glass tube carried a cotton plug *A*. Through the second hole in the rubber stopper a short glass tube *B* was passed. The upper end of this tube *B* was closed by means of a piece of rubber tubing, a screw

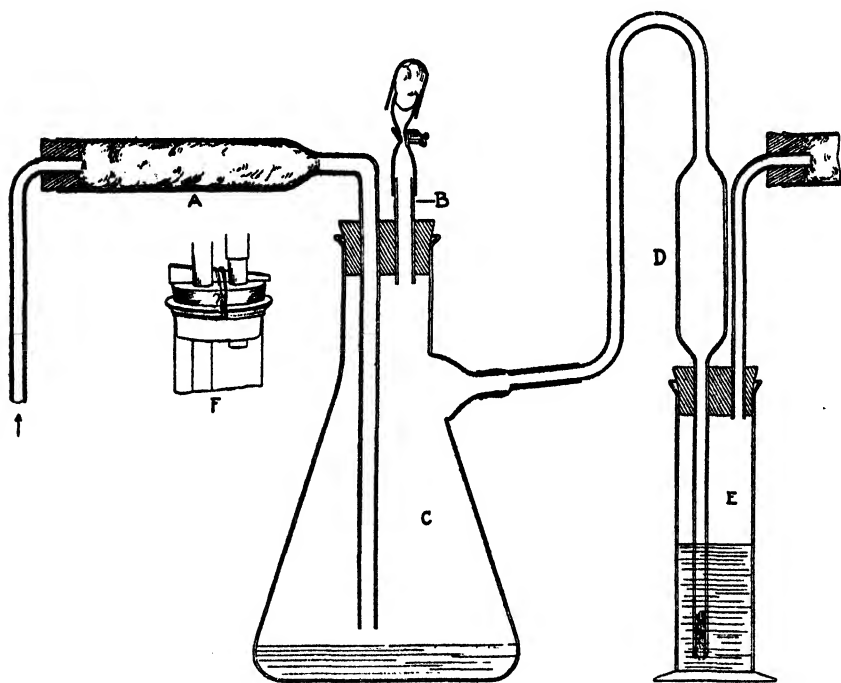


FIG. 1. FLASK FOR THE GROWTH OF AEROBIC BACTERIA AND THE ABSORPTION OF EVOLVED CARBON DIOXIDE

pinch-cock, and a cotton plug, protected by a short inverted test tube. Through tube *B*, samples were removed for analysis. The flask *C* was connected to a 50 cc. pipette, which in turn passed into the alkali flask *E*. This flask contained a solution of 30 per cent KOH. By means of a bottle aspirator, a slow current of CO₂-free air, was drawn through the system of flasks to remove evolved gases. The carbon dioxide was determined

by double titration according to the method of Scales (1920). As a check on the Scales method CO_2 was also determined by the gasometric method of Van Slyke (1917).

The cultures were connected in series and aspirated continuously with a current of CO_2 -free air. During the incubation period, usually four weeks, the entire apparatus was kept at 28°C . At irregular intervals the CO_2 receivers were removed and the CO_2 determined. After some preliminary tests, it was found that this kind of fermentation flask if carefully set up may be kept for months without contamination and without any loss of CO_2 . No attempt was made to measure gaseous products other than CO_2 .

Since all of the organisms showed an appreciable growth in the yeast water alone, each organism was grown in this medium and the carbon dioxide found was deducted from the total gas production on the yeast water plus sugar. All three cultures, *B. vulgatus*, *A. sorbose* and *A. xylinum* break down xylose with the production of large amounts of CO_2 . This substance is far in excess of all others and accounts for a large part of the sugar consumed. The data obtained from the fermentation of xylose in yeast water are given in table 2. As compared with the acid fermentation of xylose by *Lactobacilli*, the most noticeable difference is the large CO_2 production and the absence of acids. Since atmospheric oxygen is utilized in the fermentation process, a direct comparison cannot be made between the total weight of products formed and the weight of sugar consumed.

Gruner and Fraser (1921) in their study of the *Bacillus mesentericus* group and allied organisms, called attention to the action of this group on sugar media. They found that acid formation from glucose usually reaches a maximum by the second or third day and on longer periods of incubation fails to increase. Results somewhat similar to those of Gruner and Fraser were obtained with *B. vulgatus*. At first there was a small amount of acid, which in the older cultures disappeared almost completely.

Of special interest is the production of acetone and ethyl alcohol from xylose by both *B. vulgatus* and *A. xylinum*. The acetone organisms which have been isolated and studied

B. macerans (Schardinger, 1905), *B. granulobacter pectinovorum* (Speakman, 1920), *B. acetoethylicum* (Northrop, Ashe and Senior, 1919), *B. butylicus* (Ricard, 1919) are facultative anaerobic rod forms. In contrast to the organisms described in this paper these acetone forming bacteria produce acid, usually acetic or butyric, together with the neutral substances, acetone and alcohol. A recent paper by Berthelot and Ossart (1921) gives the results of acetone production by 217 cultures of bacteria isolated from soil, water and manure. Their data are substantially in agreement with the figures of table 2.

TABLE 2
Total fermentation products from xylose
Xylose at beginning 1.89 per cent. Calculated for 100 cc. of culture.

PRODUCTS	B. VULGATUS	A. SORBOSE	A. XYLINUM
	gm.	gm.	gm.
Carbon dioxide			
From xylose and yeast water.....	2.3990	2.1702	2.2223
Yeast water alone.....	0.8068	0.3457	0.8437
From xylose.....	1.3922	1.8245	1.3786
Ethyl alcohol.....	0.0784	0.0	0.3698
Acetone.....	0.0088	0.0	0.1847
Total products.....	1.4794	1.8245	1.9331
Xylose fermented.....	1.4007	1.6090	1.2530

The two organisms *A. sorbose* and *A. xylinum*, behaved somewhat differently from the description given by Brown (1886) and by Bertrand (1904). The general cultural characteristics answer to those given in the original description, but the products of fermentation were markedly different. Instead of forming arabonic acid from arabinose and xylonic acid from xylose, as described by the original investigators, the main products of fermentation were found to be carbon dioxide by *A. sorbose* and carbon dioxide, ethyl alcohol and acetone by *A. xylinum*. The formation of these two neutral products was totally unexpected and to be sure that it was not due to contamination, this experiment was repeated several times and always with the same results. *A. xylinum* produces acetone and alcohol from xylose in

large amounts. If calculated in percentage of the sugar consumed, the acetone is equivalent to 10.8 per cent, the alcohol to 18.8 per cent. These values are of special interest for comparison with those recorded in papers by Speakman (1920) for *B. granulobacter pectinovorum* and by Artzberger et. al (1920) for the acetone organism, *B. acetoethylicum*. The percentages are approximately of the same order as those reported by these investigators.

EFFECT OF AGE ON ACETONE PRODUCTION

In preliminary tests it was found that the age of the culture influences the amount of acetone. The next experiment was

TABLE 3
Effect of age of cultures on acetone and alcohol production
Calculated for 100 cc. of culture.

PRODUCT	AGE OF CULTURE	B. VULGATUS	A. XYLINUM
	days	gm.	gm.
Acetone.....	6	0.0902	0.0651
Acetone.....	20	0.0024	0.0843
Ethyl alcohol.....	6	0.1122	0.1325
Ethyl alcohol.....	20	0.0147	0.1472
Xylose fermented in twenty days		90.26 per cent	70.92 per cent

undertaken in an attempt to find out the amount of acetone at different ages of the cultures, *B. vulgatus* and *A. xylinum*. Table 3 shows the values obtained for acetone and alcohol at varying ages. These two cultures are strikingly different. *B. vulgatus* forms large amounts of acetone and alcohol in young cultures and later shows a decided decrease in these two substances, while *A. xylinum* shows only a small amount of acetone and alcohol in young cultures but increases continually to a large amount in old cultures.

THE FERMENTATION OF ARABINOSE AND XYLOSE BY *B. HERBICOLA*
AUREUM AND ORGANISMS A AND B

Because of the frequent occurrence of these three chromogenic types on green plants, their action on the pentose sugars is of particular importance. Although widely distributed in nature and easily isolated, these three forms fail to exhibit any striking physiological characteristics. No doubt these, or closely related organisms, inhabitants of living plant tissue, play an important rôle in nature.

TABLE 4
Total fermentation products from pentoses
Calculated for 100 cc. of culture.

PRODUCTS	XYLOSE AT BEGINNING 1.9765 PER CENT			ARABINOSE AT BEGINNING 1.8970 PER CENT
	Organism A	Organism B	<i>B. herbicola</i> <i>aureum</i>	<i>B. herbicola</i> <i>aureum</i>
	gm.	gm.	gm.	gm.
Carbon dioxide				
From sugar and yeast water.....	0.5810	0.6800	1.6667	2.3257
From yeast water alone.....			0.3946	0.3946
From sugar.....			1.2721	1.9311
Sugar fermented.....	0.6900	0.6850	1.5654	1.6328

The apparatus used and methods of study described in the first part of this paper were followed. After thirty days the cultures were analyzed and the results shown in table 4. These two coccus forms are slow fermenters of xylose destroying about 30 per cent, but *B. herbicola aureum* attacks the pentose sugars much more vigorously. In thirty days about 79 per cent of the xylose and 81 per cent of the arabinose was fermented. The products of fermentation are almost entirely CO₂ and a small amount of acid. From *B. vulgatus* 0.076 gram of non-volatile acid calculated as lactic was obtained for 100 cc. of medium.

SUMMARY

This report deals with the fermentation of the pentose sugars by certain aerobic bacteria, viz., *B. vulgaris*, *A. sorbose*, *A. xylinum*, *B. herbicola aureum* and two yellow coccus forms designated Organism A and B. The rate of fermentation of xylose, and in certain cases arabinose, by these aerobic forms is much slower than is noted with the facultative anaerobic bacteria.

The products of the fermentation of the pentose sugars depends upon the kind of organism and the ratio of the products on the age of the culture. *B. vulgaris* ferments xylose with the production of acetone, ethyl alcohol, carbon dioxide and a small amount of fixed acids. As the culture becomes older the ratio of acetone and ethyl alcohol to carbon dioxide decreases. The maximum amount of acetone and alcohol is found about the sixth day after inoculation.

In the case of *A. xylinum* an increase in the age of the culture is accompanied by an increase in the yield of acetone and alcohol. Although in different proportions the substances obtained in the breaking down of xylose, *A. xylinum* are the same as noted with *B. vulgaris*. *A. sorbose*, *B. herbicola aureum* and the two coccus forms ferment the pentoses slowly and the chief end product is carbon dioxide and a trace of acid.

It may be concluded that in nature many of the commonly occurring aerobic forms of bacteria attack the pentose sugars and form as the chief end products, neutral substances such as acetone and ethyl alcohol, and carbon dioxide.

An apparatus for conducting the fermentations under aerobic conditions is described.

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THE RELATION BETWEEN CHEMICAL COMPOSITION OF PEPTONES AND HYDROGEN SULPHIDE PRODUCTION BY BACTERIA

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In a paper recently published in this journal (Tilley, 1923) the writer showed that variations in hydrogen sulphide production by bacteria in lead acetate agar may be due on the one hand to differences in the hydrogen sulphide producing power of different strains of the same organism and on the other hand to the use of different varieties of peptone in the lead acetate agar. As explained in that paper the writer had expected to find that the value of the various peptones for H_2S production depended upon the amount of cystin present, just as the value of peptones for indol production depends upon the tryptophan content (Tilley, 1921). Preliminary experiments, however, indicated that the problem was more complicated than at first supposed so further work was undertaken, the results of which are reported in the present paper.

EXPERIMENTAL WORK

In view of the great complexity of the composition of peptones it was not considered advisable to attempt to make complete and exact analyses of the various peptones used. For the purposes of the work here reported it was thought sufficient to ~~ascertain~~ roughly the approximate amounts of "unoxidized," "partly oxidized" and "oxidized" sulphur in the six peptones employed in the work reported in the previous paper.

The term "unoxidized sulphur" will be used in this paper to denote sulphur as it occurs in proteins and protein derivatives

loosely combined with carbon and hydrogen. Upon heating with caustic alkali hydrogen sulphide is liberated and as it is detected by means of lead acetate solution or lead acetate paper this form of sulphur is often called "lead blackening sulphur." Cystin is an example of this form of sulphur. The term "partly oxidized sulphur" will be used to denote sulphur combined in such a form that upon distillation with phosphoric acid sulphur dioxide is liberated. The term "oxidized sulphur" will be used to denote sulphur as it occurs in sulphates or other similar compounds.

Unoxidized sulphur was estimated in the following manner: 5 cc. of a strong (1:1) solution of potassium hydroxide were

TABLE 1

Relation between chemical composition of peptones and H₂S production by bacteria

PEPTONE NUMBER	UNOXIDIZED SULPHUR	PARTLY OXIDIZED SULPHUR	OXIDIZED SULPHUR	H ₂ S BY BACTERIA
1	+	+	+	+
2	-	+++	+++	+++
3	++	-	-	-
4	++	++	-	++
5	+++	+	+++	++
6	-	+++	+++	+++

- = none; + = small amount; ++ = moderate amount; +++ = large amount.

mixed with an equal quantity of a filtered 3 per cent solution of peptone in water or in 20 per cent hydrochloric acid; a few drops of a 10 per cent aqueous solution of lead acetate were added and the mixture boiled vigorously. The relative amounts of unoxidized sulphur present were estimated roughly by the amount of blackening which resulted.

The estimation of partly oxidized sulphur was made by Mr. R. R. Henley of this Division, to whom I am also much indebted for advice and assistance so far as the chemical aspects of this work are concerned. The details of the method employed may be found in Bureau of Chemistry Bulletin No. 107 (1908). Oxidized sulphur was estimated by precipitation with barium chloride in an acid solution.

The relation between chemical composition of the various peptones, as shown by the previously described tests, and hydrogen sulphide production by bacteria in lead acetate agar containing these peptones is shown in table 1. It should be clearly understood that the relative amounts indicated are only rough approximations. In order to avoid any suggestion that the differences shown represent exact quantitative results arbitrary symbols are employed instead of any statement of amounts.

The results shown in table 1 indicated that partly oxidized sulphur is the form of sulphur which yields the largest amount of hydrogen sulphide. Further experiments were, however,

TABLE 2
Relative values of oxidized and partly oxidized sulphur for H₂S production

ORGANISM AND STRAIN	H ₂ S PRODUCTION BY BACTERIA IN MEDIA CONTAINING		
	No added sulphur	Ammonium sulphate	Sodium thiosulphate
<i>Proteus</i> 4.....	+	+	+++
<i>Bact. suispestifer</i> 416.....	+	+	+++
<i>Bact. suispestifer</i> 420.....	+	+	+++
<i>Bact. enteritidis</i> G 4.....	+	+	+++
<i>Bact. typhosum</i> A.....	+	+	++
<i>Bact. typhosum</i> C.....	+	+	++
<i>Bact. typhosum</i> D.....	—	—	—

— = no H₂S; + = small amount; ++ = moderate amount; +++ = large amount. Results shown are after twenty-four to forty-eight hours incubation.

undertaken to determine more exactly the relative value of the different forms of sulphur for hydrogen sulphide production. In these experiments various sulphur compounds were added to lead acetate agar and observations made upon the relative amounts of hydrogen sulphide produced by various bacteria.

In the first place an attempt was made to show the relative values of oxidized sulphur in the form of sulphate and partly oxidized sulphur in the form of thiosulphate. These compounds were added to lead acetate agar prepared according to the directions of Jordan and Victorson (1917) except that the reaction was adjusted to pH 7.2 instead of to +1 to phenolphthalein. The amount of sulphate or thiosulphate added was 0.25 per cent.

The peptone used in the preparation of the lead acetate agar was peptone 1 (see table 1). The results are given in table 2.

The results shown in table 2 indicate that oxidized sulphur in the form of sulphate does not yield any appreciable amount of hydrogen sulphide while partly oxidized sulphur in the form of thiosulphate seems to be especially suitable for hydrogen sulphide production by bacteria.

TABLE 3

Comparative effect of different compounds containing partly oxidized sulphur

ORGANISM AND STRAIN	H ₂ S PRODUCTION BY BACTERIA IN MEDIA CONTAINING		
	No added sulphur	Sodium thiosulphate	Sodium sulphite
<i>Bact. suipestifer</i> 360.....	—	—	+++
<i>Bact. suipestifer</i> 416.....	—	+++	+
<i>Bact. coli</i> C.....	—	—	+
<i>Bact. cloacae</i> T 2.....	—	—	+
<i>Proteus</i> 4.....	—	+++	+
<i>Proteus</i> 11.....	—	+	—
<i>Bact. typhosum</i> A.....	—	+++	+++
<i>Bact. typhosum</i> K 110.....	—	—	++
<i>Bact. dysenteriae</i> Y.....	—	—	+
<i>Bact. dysenteriae</i> Shiga.....	—	—	—
Paratyphoids.....	—	—	—
A. K A 28.....	—	—	++
B. K B 31.....	—	+++	+++
C. K C 1.....	—	+++	+++

— = no H₂S; + = small amount; ++ = moderate amount; +++ = large amount.

In the remaining experiments instead of following the directions of Jordan and Victorson, lead acetate agar was prepared by adding lead acetate solution to ordinary beef infusion agar containing 1 per cent of peptone 1. In the experiment shown above in table 3, sodium thiosulphate or sodium sulphite was added to this medium in the proportion of 0.25 per cent.

The results shown above indicate that, while both sodium thiosulphate and sodium sulphite readily yield hydrogen sulphide, sodium thiosulphate, on account of clearer distinctions between organisms and strains, would give the better results when used

in a culture medium intended for diagnostic purposes to distinguish between different species and strains of bacteria.

In table 4 there are shown the results of an experiment with representatives of the two classes of organic compounds containing sulphur designated by Hawk (1918, p. 108) as unoxidized and oxidized. Cystin was selected as containing unoxidized organic sulphur and taurin as containing oxidized organic sulphur.

TABLE 4^a

Comparative value of different organic sulphur compounds for H₂S production

ORGANISM AND STRAIN	H ₂ S PRODUCTION BY BACTERIA IN MEDIA CONTAINING		
	No added sulphur	Cystin	Taurin
<i>Bact. suipestifer</i> 360.....	—	+	—
<i>Bact. suipestifer</i> 416.....	—	++	—
<i>Bact. coli</i> C.....	—	+++	—
<i>Bact. cloacae</i> T 2.....	—	++	—
<i>Proteus</i> 4.....	—	+++	—
<i>Proteus</i> 11.....	—	+++	—
<i>Bact. typhosum</i> A.....	—	++	—
<i>Bact. typhosum</i> K 110.....	—	+	—
<i>Bact. dysenteriae</i> Y.....	—	+	—
<i>Bact. dysenteriae</i> Shiga.....	—	+	—
Paratyphoids.....	—		
A. K A 28.....	—	++	—
B. K B 31.....	—	+++	—
C. K C 1.....	—	+++	—

— = no H₂S; + = small amount; ++ = moderate amount; +++ = large amount.

The technique employed was similar to that used in experiment III except that the amount of cystin used was 0.1 per cent and special technique was employed in adding it to the culture medium. The cystin was partly dissolved and partly suspended in N/10 HCl and after sterilization in the autoclave was added to the culture medium with a sterile pipette. Just previous to this there was added enough sterile N/10 NaOH to neutralize the N/10 HCl in which the cystin was carried, thus leaving the reaction of the culture medium as far as possible unchanged.

The results given in table 4 indicate that taurin is not utilized by bacteria for H_2S production while cystin gives rise to an abundant production of H_2S , so much so that differences between the various species and strains are largely obliterated. This, and the fact that organisms usually classed as "lead negative" are rendered "lead positive," make it inadvisable to use cystin to correct deficiencies in the sulphur content of peptones used in lead acetate agar.

The results of table 4 taken together with those of table 1 bring up a very interesting question. In table 4 we see that unoxidized sulphur in the form of cystin yields a large amount of H_2S and yet in table 1 we see that although peptone 3 contains apparently as much unoxidized sulphur as any of the peptones except no. 5, no appreciable amount of H_2S is formed in lead acetate agar made with peptone 3.

The most plausible explanation of this discrepancy would seem to be that the unoxidized sulphur of the various peptones is not all free cystin.

In order to test this explanation the six peptones previously employed were examined for free cystin in the following manner, the technique employed being based on that given by Hawk (1918, p. 87) for the separation of cystin formed by the action of hydrochloric acid on wool; 5 grams of peptone were dissolved in 25 cc. of 20 per cent hydrochloric acid, the solution being kept cool to avoid hydrolytic action as far as possible, and the resultant solution filtered. To this filtered solution sodium acetate was added in excess, as shown by a negative reaction for mineral acid with Congo red. Whatever precipitate was formed was separated out by filtration through a hardened filter paper and then dissolved in hot dilute (5 per cent) hydrochloric acid. Then this dissolved precipitate, and also the filtrate from the original solution, were tested for unoxidized sulphur by the method already given in the first part of the paper. Free cystin being insoluble in acetic acid should appear in the precipitate while any unoxidized sulphur found in the filtrate would be regarded as something other than free cystin. The results are shown in table 5.

The results shown below indicate that none of the peptones

except no. 5 contain any really appreciable amount of free cystin. The presence of cystin in no. 5 is no doubt the reason for the results shown in table 1 where it is seen that although peptone 5 contains very little partly oxidized sulphur it yielded a fairly large amount of hydrogen sulphide. The fact that the cystin served to bring up the amount of hydrogen sulphide only to a moderate degree is presumably due to its relative insolubility. In spite of the large amount in the peptone only a small amount was dissolved by the culture media.

TABLE 5

Relative amounts of free cystin and other unoxidized sulphur in peptones

PEPTONE NUMBER	UNOXIDIZED SULPHUR	
	Precipitate	Filtrate
1	None	Present
2	None	Present
3	None	Present
4	A trace	Present
5	Present	Present
6	None	Present

DISCUSSION

It is interesting, in the first place, to note that the results shown in tables 2, 3 and 4 agree very well with those reported by Myers (1920) and by Sasaki and Otsuka (1912). These investigators worked with various sulphur compounds in fluid media containing no peptone but their results correspond very closely to those obtained by the writer with the same compounds in lead acetate agar.

It seems evident from the experimental results herein reported that commercial peptones contain unoxidized, partly oxidized and oxidized sulphur in varying proportions. On account of differences in the amount and availability of these various forms of sulphur in the peptones it is inevitable that variable results should be obtained with lead acetate agar made with the different peptones unless precautions are taken to prevent such results.

Variable results could be avoided by testing the peptones

employed, either by chemical methods similar to those described in this paper, provided the necessary apparatus is available, or by actual trial in media with known strains of known organisms.

The use of sodium thiosulphate as a usual ingredient of lead acetate agar would, however, obviate the necessity for testing the peptone employed and in the writer's judgment, the results obtained would be entirely comparable with those usually obtained with the best peptones. It is quite possible that with organisms other than those used by the writer, or with different strains of the same organisms, this might not hold true but on the other hand the use of a poor peptone will give wholly misleading results, and even with the best peptones there may be variations in the composition of different samples and consequent variations in the results obtained.

It is therefore recommended that sodium thiosulphate be used as a regular ingredient of lead acetate agar. If used in a synthetic medium containing no other source of sulphur it would no doubt give clearer distinctions between different organisms and strains than in a peptone medium. But so far as the writer is aware, there is no synthetic medium which will support as vigorous growth by as many different organisms as the usual peptone media.

In the absence of such a synthetic medium it seems advisable to use lead acetate agar prepared according to the directions of Jordan and Victorson (1917) except for the following changes: the use of 1 per cent instead of 3 per cent of peptone, adjustment of reaction by the hydrogen ion method instead of by titration, and the addition of sodium thiosulphate. The smaller amount of peptone is sufficient to support the growth of bacteria and would lessen possible interference by sulphur compounds already present in the peptone. The writer has in his own work adjusted the reaction to pH 7.2 and used 0.25 per cent of sodium thiosulphate.

CONCLUSIONS

1. Commercial peptones have been shown to contain unoxidized, partly oxidized and oxidized sulphur in varying proportions.

2. Bacteriological tests with media containing various sulphur compounds showed that no hydrogen sulphide was liberated by bacteria from compounds containing oxidized sulphur while it was given off freely from those containing partly oxidized sulphur.

Unoxidized sulphur in the form of cystin yielded an abundance of hydrogen sulphide but experimental evidence indicated that the unoxidized sulphur of commercial peptones may consist largely of some compound, or compounds, other than cystin and not utilized by bacteria for the production of hydrogen sulphide.

3. On account of the qualitative and quantitative differences in the sulphur content of the various commercial peptones, there are resulting variations in hydrogen sulphide production by bacteria in media containing these peptones.

4. To insure uniform results in testing hydrogen sulphide production by bacteria in lead acetate agar the peptone used should be suitable for the purpose, as shown by chemical or bacteriological tests; or, preferably, sodium thiosulphate should be used as an ingredient of the lead acetate agar.

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GELATIN LIQUEFACTION BY BACTERIA

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The liquefaction of gelatin is generally recognized and employed as a fundamental criterion for the differentiation of bacterial species. Unfortunately the methods in vogue for observing this property are crude and unreliable. Usually nutrient gelatin is inoculated by stabbing, kept at a temperature below its gelation point, and compared with controls to detect any liquefaction. Measurement of the rate of liquefaction is sometimes attempted by inoculating the entire surface of a tube of gelatin and recording the depth of liquid formed, after different periods of storage, but comparable results are rarely obtained.

The first step in the liquefaction of gelatin is peptization or transformation from the gel to the sol state; from a very viscous to a more fluid condition. Davis and Oakes (1922), in a recent paper on the physical characteristics of gelatin solutions have shown that the transformation from the gel to the sol state in 4 per cent gelatin solution takes place at 38.03°C. Above this temperature, the viscosity remains constant on ageing or decreases if the temperature is sufficiently high to cause hydrolysis. Below this transition point, viscosity increases with age.

At the meeting of the Society of Bacteriologists in 1919, William M. Clark (1920) reported some observations on proteus gelatinase in which he determined the solidification time by a modification of the method of Palitzsch and Walbum. Unfortunately the complete paper has not been published and the abstract does not give full details.

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We feel that further observations on viscosity changes are much needed and would be very instructive. It will be shown in this paper that in autoclaved gelatin, stored at 22°C., the viscosity may change but little for two or three days, after which it increases markedly for at least a week or ten days. Obviously a gelatinolytic organism may bring about liquefaction quite rapidly when inoculated into freshly prepared gelatin, and much more slowly if introduced into the same medium ten days or two weeks later, for in the latter instance more of the gel form must be transformed to the sol state to make liquefaction evident. Some method which could be employed over a wide range of temperature for measuring the rate of liquefaction of gelatin by bacteria is sorely needed.

In this preliminary paper are recorded some observations on the change in viscosity and formol (Sörensen) titration of gelatin subjected to bacterial decomposition.

EXPERIMENTS WITH LOW TEMPERATURE ORGANISMS

Seven organisms isolated from milk were employed. These strains grew very well at room temperature, poorly at 37°C., and not at all at 39 to 40°C. The characteristics of the organisms are indicated in the following table, kindly furnished us by Miss Lulu Soppeland of the Iowa Engineering Experiment Station who will report a more detailed study in the near future.

Medium. The gelatin medium employed consisted of

Peptone (Difco).....	1.0 gram
Gelatin (Difco).....	35.0 grams
Water (distilled).....	1000 cc.

This was heated at 60 to 65°C. till dissolved, then further heated in a double boiler for 15 minutes, the reaction adjusted to pH 8.0, loss due to evaporation made up, and heated an additional 15 minutes, then filtered through cotton and flannel, tubed (25 cc. quantities) and sterilized at 15 pounds for fifteen minutes. The medium was allowed to cool at room temperature and the following day (twenty-four hours) inoculations were made.

LAB. NO.	GENERAL CHARACTERISTICS	S. A. B. GROUP NO.
2019	Gram negative; short rods; singly or short chains; non-motile; spores not found; gelatin not liquefied; greenish pigment on agar	222.3331433
2011	Gram positive; irregular shapes; budding frequent; spores not found, non-motile; gelatin slowly liquefied. (Mold like)	221.2323913
2060	Gram negative; short rods; singly or short chains; spores not found; non-motile; gelatin liquefied slowly; orange pigment	221.3333633
2053	Gram positive; short rods singly or short chains; spores not found; non-motile; gelatin liquefied; orange yellow pigment	221.2323623
1021	Gram positive; short rods singly or short chains; spores not found; motile; gelatin liquefied	221.1112811
1059	Gram positive; short rods; singly or short chains; spores not found motile; gelatin liquefied; yellow pigment	221.2323512
1045	Gram positive; short rods; singly or short chains; non-spore-forming; non-motile; gelatin liquefied; yellow pigment	221.2323513

The inoculum consisted of 1 cc. of a twenty-four-hour broth culture of the test organism and incubation was at 22°C. Control tubes were inoculated with 1 cc. of sterile broth.

Peptone water (0.1 per cent) was similarly inoculated to afford a correction for the peptone in the gelatin medium. Each organism was inoculated into 5 tubes gelatin and peptone water and at stated intervals one tube of each medium was removed from the incubator and examined.

Viscosity measurements were made at 25.6°C. by observing the time of discharge of a given volume in an Ostwald viscosimeter. In the following table the viscosity is expressed in terms of water, at the same temperature, as unity. The time of flow for water was 88.5 seconds.

Formol titrations were carried out in the following manner: To 20 cc. of distilled water in an evaporating dish were added 5 cc. of gelatin (or peptone) medium and the reaction adjusted

TABLE 1

Effect of period of incubation on formol (Sørensen) titration and viscosity

INCUBATION (22°C.)	FORMOL TITRATION				VISCOSITY (25.8°C.) REFERRED TO H ₂ O
	Peptone	Peptone gelatin	Gelatin	Increase from gelatin	
Controls					
<i>days</i>					
0	0.13	1.46	1.33*		5.38
1	0.13	1.46	1.33		5.38
3	0.14	1.35	1.21		5.14
5	0.14	1.29	1.15		12.67
7	0.13	1.42	1.29		19.15
12	0.13	1.48	1.35		†
Organism 2019					
0	0.13	1.46	1.28		5.38
1	0.26	1.51	1.25	-0.03	5.29
3	0.23	1.36	1.13	-0.15	5.21
5	0.26	1.43	1.17	-0.11	9.82
7	0.18	1.60	1.42	+0.14	13.66
12	0.27	1.53	1.26	-0.02	†
Organism 2011					
0	0.13	1.46	1.28		5.38
1	0.16	1.50	1.36	0.08	3.51
3	0.19	1.70	1.51	0.23	2.91
5	0.26	1.79	1.54	0.26	2.05
7	0.21	2.66	2.45	1.17	1.75
12	0.34	2.73	2.39	1.11	1.27
Organism 2060					
0	0.13	1.46	1.28		5.38
1	0.17	1.54	1.37	0.09	4.08
3	0.17	1.90	1.73	0.45	3.66
5	0.24	1.88	1.64	0.38	4.02
7		2.11	(1.84)	0.56	3.58
12	0.32	4.28	3.96	2.68	1.29

* Employed 1.28 as the average of controls for correction.

† Too viscous to ascertain.

Formol titration expressed in terms of N/1 NaOH required per 100 cc. of medium for neutralization after treatment with formaldehyde.

TABLE 1—Continued

INCUBATION (22°C.)	FORMOL TITRATION				VISCOSITY (25.6°C.) REFERRED TO H ₂ O
	Peptone	Peptone gelatin	Gelatin	Increase from gelatin	
Organism 2053					
<i>days</i>					
0	0.13	1.46	1.28		5.38
1	0.17	1.58	1.41	0.13	2.61
3	0.22	1.81	1.59	0.31	2.08
5	0.30	1.91	1.61	0.33	1.92
7	0.25	2.57	2.32	1.04	1.76
12	0.32	2.99	2.67	1.39	1.32
Organism 1021					
0	0.13	1.46	1.28		5.38
1	0.36	2.24	1.88	0.60	1.80
3	0.37	3.61	3.24	1.96	
5	0.41	4.83	4.42	3.14	1.37
7	0.41	5.78	5.37	4.09	1.38
12	0.50	7.64	7.14	5.86	1.20
Organism 1059					
0	0.13	1.46	1.28		5.38
1	0.34	2.42	2.08	0.80	1.49
3	0.50	5.99	5.49	4.21	1.35
5	0.79	9.20	8.41	7.13	1.27
7	0.83	13.61	12.78	11.50	1.21
12	0.78	18.18	17.40	16.12	1.16
Organism 1045					
0	0.13	1.46	1.28		5.38
1	0.27	2.16	1.89	0.16	1.54
3	0.40	4.16	3.76	2.48	1.34
5	0.62	6.45	5.83	4.55	1.34
7	0.72	9.48	8.76	7.48	1.33
12	0.74	14.00	13.26	11.98	1.17

to neutrality to phenolphthalein, 10 cc. of a 50 per cent formalin (neutral to phenolphthalein) was then added, and after ten minutes the sample was titrated with $N/50$ NaOH. In the tables the formol titration is expressed in terms of cc. $N/1$ NaOH per 100 cc. of medium or per cent normality of acid liberated by the formaldehyde. Some difficulty was encountered in

determining the exact end point, but in general the differences observed with the various cultures were so great that this end point error (0.1 to 0.15 cc.) may be disregarded.

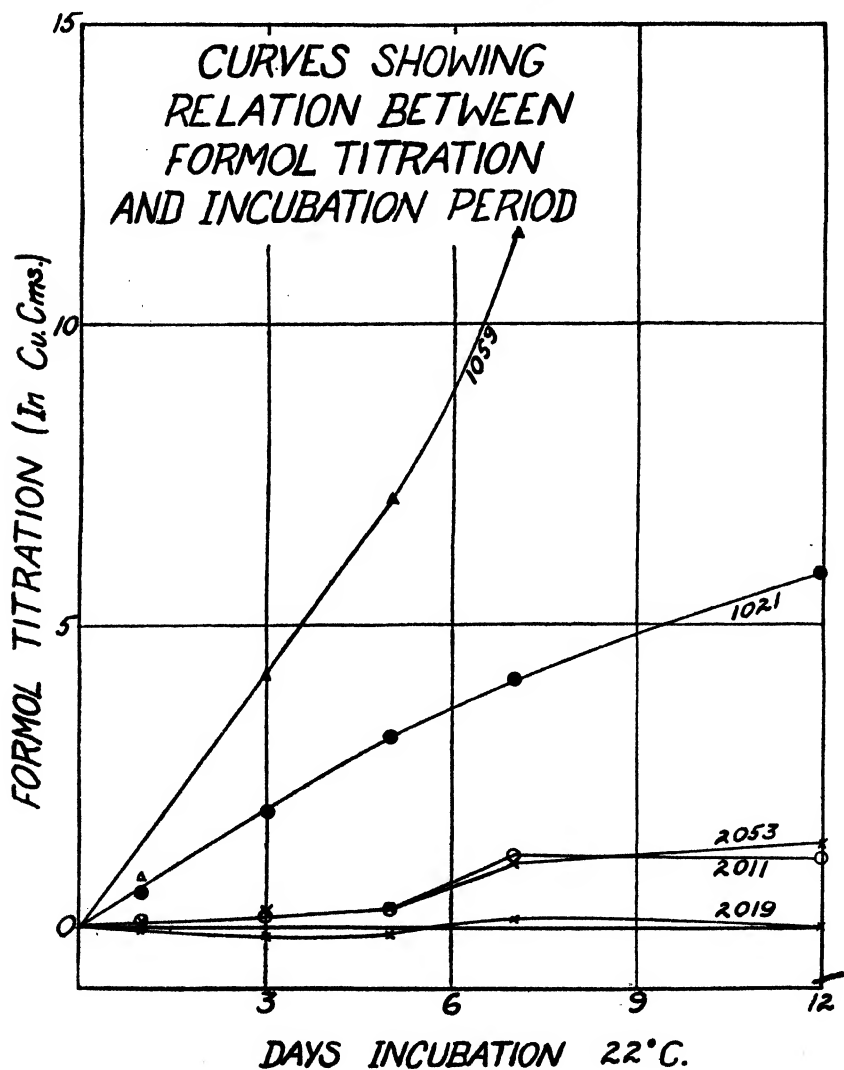


FIG. 1. INCREASE IN FORMOL TITRATION (CC. N/1 NaOH REQUIRED PER 100 CC. MEDIUM) ...

The results are detailed in table 1, and in figures 1 and 2 are shown the changes in relative viscosity and formol titrations brought about by the growth of the various bacteria studied.

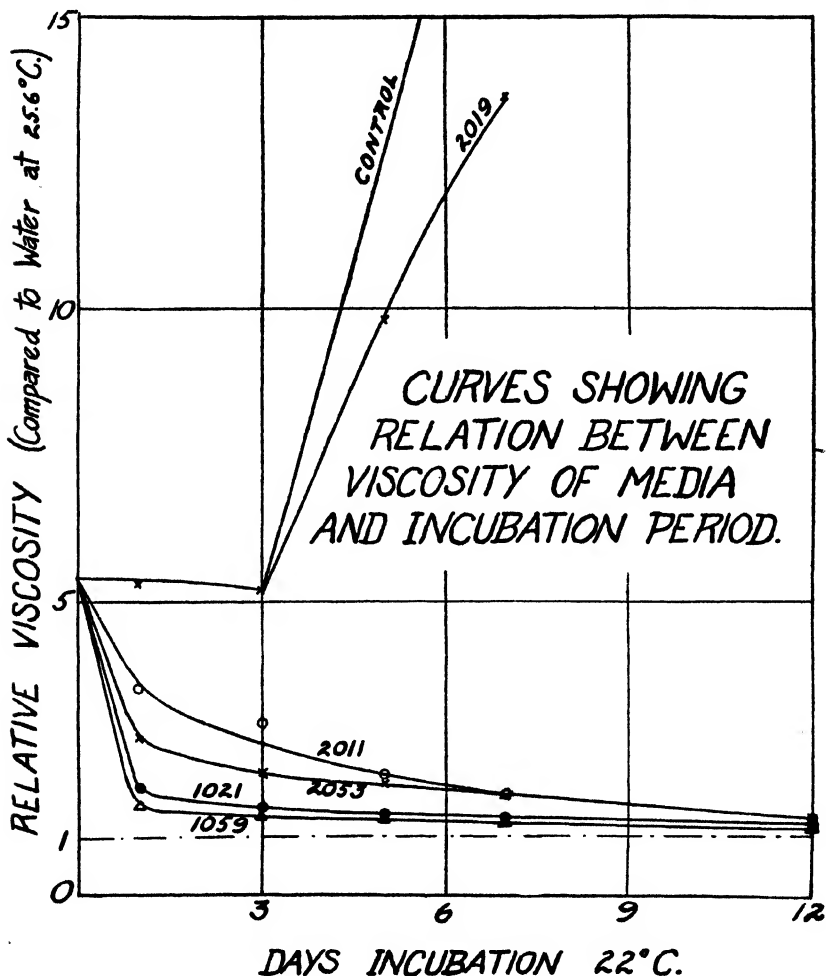


FIG. 2. CHANGE IN VISCOSITY OF PEPTONE-GELATIN

The viscosity and formol titration changes indicate that, with respect to their action on gelatin, bacteria may be subdivided into three groups as follows:

1. Gelatin not hydrolized; liquefaction negative.
2. Gelatin partially hydrolized and liquefied. Subsequent decomposition slow if any; accompanied by slight increase in formol titration.
3. Gelatin more completely hydrolized, and liquefied. Subsequent decomposition, rapid; accompanied by marked increase in formol titration.

As an example of the non liquefying type we have organism 2019. The relative viscosity of the medium (0.1 per cent peptone—3.5 per cent gelatin) increased from 5.38 to 9.38 in five days, to 13.60 after seven days incubation, and after twelve days the gelatin was solidified. There was a slight rise in formol titration, but after correcting for the peptone, the increase, which may be ascribed to the gelatin proper, became a negative quantity. This is due probably to the fact that in peptone water the organism grew throughout the medium whereas in the peptone-gelatin medium growth was restricted to the upper layers.

Organisms 2011, 2053 and 2060 are representative of the second type. There is a distinct drop in the viscosity twenty-four hours after inoculation (from 5.38 to 3.51, 4.08 and 2.62 respectively) with practically no increase in the formol titration. On further incubation the viscosity continues to decrease and the formol titration rises slowly.

With organisms 1021, 1059 and 1045, the change in viscosity is much more marked falling to 1.86, 1.49 and 1.54 respectively in twenty-four hours. The rise in the formol titration is relatively rapid, the increase in one day being as great as was observed in five to seven days among the strains of the previously described liquefying group.

The results are quite in accord with the statement of Berman and Rettger (1918) that "the power of an organism to liquefy gelatin is not necessarily accompanied by the ability to decompose the gelatin and seize upon it as food."

To ascertain whether the liquefaction is independent of the presence of living microorganisms, the following experiment was carried out.

Eight tubes of the above batch of peptone-gelatin which had solidified were heated for thirty minutes at 55°C. to convert to the sol form, and then cooled to 22°C.

To each of four tubes were added 2.5 cc. of 5 per cent phenol (giving a concentration of 0.5 per cent phenol) and to each of the remaining 4 gelatin tubes were added 2.5 cc. of broth. Two of the phenolated and two of the ordinary gelatin tubes were inoculated with 1 cc. of a forty-eight-hour broth culture of organism 1059, and to the remaining (control) tubes was added 1 cc. of broth so as to maintain the concentration of gelatin as equal as possible. All were incubated at 22°C. The results are indicated in table 2.

TABLE 2

Effect of period on incubation and presence of phenol (0.5 per cent) on formol titration and viscosity of peptone gelatin

ORGANISM	INCUBATION (22°C.)	NOT PHENOLATED		PHENOLATED	
		Formol titration	Viscosity	Formol titration	Viscosity
	<i>days</i>				
None {	1	1.52	4.95	1.56	3.34
	6	1.54	6.58	1.56	4.90
1059 {	1	3.04	1.50	1.65*	1.62
	6	10.44	1.18	1.71	1.31

* Corrected for formol titration of 1 cc. broth inoculum (0.17 cc.)

There was no evidence at any time of growth in the phenolated medium. The liquefaction was therefore due to the presence of enzymes, secreted by the microorganisms in question. Considering the formol titration it is clearly evident that in the absence of bacterial growth (phenolated gelatin) there was practically no increase, whereas the increase was very considerable in the non phenolated tube. These results are in accord with those obtained by Kendall and his associates (1922) on studies with *Proteus*. They observed that liquefaction of gelatin by the bacteria-free enzyme was accompanied by a slight increase in ammonia and amino acids (formol titration), whereas in the presence of the viable bacteria, the ammonia content rose very markedly.

The slight increase in the formol titration observed in our experiments with phenolated gelatin is taken to represent the increase in ammonia and amino acids accompanying the enzymic

hydrolysis of gelatin. The marked rise obtained with the actively growing bacteria is most likely due to liberation of ammonia due to intracellular deaminization. Further work on this point is contemplated.

SUMMARY

The change in viscosity of a gelatin medium and simultaneous rate of increase of the "formol titration" was observed with 7 organisms.

The viscosity was found to drop before the formol titration begins to rise. The rate of increase in formol titration serves to distinguish two types of gelatin liquefiers.

A standardized method for ascertaining the change in viscosity of gelatin culture media should be far superior to the present methods of detecting gelatin liquefaction. A temperature of 40°C. which is slightly above the gelation point is suggested as desirable for this purpose and is now under investigation.

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VARIATION IN THE LIMITING HYDROGEN-ION CONCENTRATIONS OF STREPTOCOCCI

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The limiting hydrogen-ion concentration produced by streptococci in fluid media has been suggested as a possible means of differentiating between bovine and human strains. Some authors have called attention to a parallelism between the limiting hydrogen-ion concentration and pathogenicity of streptococcus strains.

HISTORICAL

The literature on the limiting hydrogen-ion concentration of streptococci has been reviewed in another place (Arnold, 1921).

TECHNIC

The strains of streptococci studied were obtained from the throats of diphtheria patients. All cultures were centrifuged well before the hydrogen-ion determination was made. Each strain was plated out twice on blood agar and colonies picked before we began the limiting hydrogen-ion determinations. Hiss-serum water was prepared in the usual manner. For the past three years we have maintained our stock cultures of streptococci on Holman's cooked meat medium (Holman, 1919). All of the media used in this work were made up in large quantities and the same original lot was used throughout the entire course of the experiments.

EXPERIMENTS

Table 1 gives the results of the comparison of the limiting hydrogen-ion concentration of 12 hemolytic and 4 viridans

strains of streptococci on 1 percent glucose Hiss-serum water and 1 percent glucose Huntoon hormone bouillon. The initial reaction of both media at the time of the inoculation was pH 7.45.

Table 2 is a record of the results obtained by using Holman's cooked meat medium as a stock culture medium for the streptococcus strains for a month. At periods of about a week apart

TABLE 1

STRAIN	LIMITING H-ION CONCENTRATION AFTER GROWING TWENTY-FOUR HOURS ON 1 PERCENT GLUCOSE. HISS SERUM WATER	LIMITING H-ION CONCENTRATION AFTER GROWING TWENTY-FOUR HOURS ON 1 PERCENT HUNTOON'S HORMONE BOUILLON	
Hemolytic			
H2H	5.5	5.1	0.4
-H17H	5.5	5.3	0.2
H16H	5.5	5.3	0.2
H3H	5.5	5.15	0.35
-H14H	5.4	5.4	0
H11H	4.7	4.6	0.1
H8H	5.2	5.2	0
H6H	5.2	5.0	0.2
H9H	5.4	5.4	0
-H10H	5.2	5.0	0.2
H14H	5.7	5.3	0.4
-H7H	5.4	5.1	0.3
Viridans			
H10V	5.3	5.0	0.3
HSV	5.4	5.2	0.2
H15B	5.1	5.1	0
H12V	5.0	5.2	-0.2

the strains were transferred from Holman's meat medium to glucose hormone bouillon and the limiting hydrogen-ion concentration determined after twenty-four hours' growth. These same strains were transplanted every other day during the month on plain hormone bouillon, and the limiting hydrogen-ion concentration was determined after twenty-four hours growth on glucose hormone medium. The object of this experiment was to see what effect the Holman's cooked meat medium might have on the

streptococci in reference to their limiting hydrogen-ion concentration in broth cultures and to see if there were any changes in the hemolytic action of the strains of streptococci—in stock cultures.

To illustrate the variations in the limiting hydrogen-ion concentration of streptococci in wounds, as compared with the sugar fermentation of the strains, we wish to record two streptococci wound flora charts. These are typical examples of wound streptococci flora; the full report, including some 50 cases, will be published later in a clinical journal.

DISCUSSION

The hemolytic streptococci have been found by most investigators to be more constant in their cultural and also in their serological characteristics (Kinsella, 1918; Dochez, Avery and Lancefield, 1919; Tunncliffe, 1920; etc.) than the viridans types of streptococci.

The results recorded in table 2 bear this out as a general rule in regard to their cultural characteristics on the media used. The 2 strains of hemolytic types, D32H and D24H, which differed from the 12 strains in their limiting hydrogen-ion concentration after growth on Holman's cooked meat medium for a month, produced a green zone on plating from this medium, while the same strains transplanted daily on glucose bouillon remained hemolytic when plated at the same time.

The viridans strains are not so constant in their limiting hydrogen-ion concentration as the hemolytic strains. The change from viridans to hemolytic types is more pronounced than the change from hemolytic to viridans types.

If the changes produced in the environment of a streptococcus colony on a blood-agar plate is the result of its metabolic function, it is not surprising that with changes of a chemical and physical nature in the medium, one should find a change in the action of the diffusible substances from the growing colony on its surrounding medium.

If a strain of streptococcus is transplanted daily for thirty days on Huntoon hormone bouillon and compared with the

TABLE 2
Limiting hydrogen-ion concentration of streptococci

ISOLATED MAY 1, 1922	Hemolytic										Changed to viridans		Changed to viridans	
	1 PER CENT GLUCOSE HORMONE ME- DUM MAY 4, 1922	TRANSPICANT DAILY ON HORMONE MEDIUM MAY 3 UNTIL MAY 8; ON CENT HORMONE MEDIUM TO MAY 9	ON HOLMAN MEAT MEDIUM MAY 4 TO MAY 8; ON 1 PER CENT GLU- COSE HORMONE BOUILLON MAY 8 TO MAY 9	TRANSPICANT DAILY ON HORMONE BOUILLON MAY 3 TO MAY 19; ON CENT GLUCOSE HORMONE MEDIUM MAY 19 TRANSPLANTED ON 1 PER CENT GLUCOSE HORMONE MEDIUM TO MAY 20; 1 PER CENT HORMONE BOUILLON MAY 19 TO MAY 20	ON HOLMAN MEAT MEDIUM MAY 4 TO MAY 26; 1 PER CENT GLU- COSE HORMONE BOUILLON MAY 26 TO MAY 28	TRANSPICANT DAILY ON HORMONE BOUILLON MAY 3 TO MAY 25; ON CENT GLUCOSE HORMONE MEDIUM MAY 25 TRANSPLANTED ON 1 PER CENT GLUCOSE HORMONE MEDIUM TO MAY 26	ON HOLMAN MEAT MEDIUM MAY 4 TO MAY 26; ON 1 PER CENT GLU- COSE HORMONE BOUILLON MAY 26 TO MAY 28	JUNE 3, PLATED FROM 1 PER CENT HORMONE BOUILLON ON 1 PER CENT GLUCOSE HORMONE BOU- ILLON JUNE 4 TO JUNE 5	JUNE 3, PLATED FROM HOLMAN MEAT MEDIUM ON BLOOD AGAR. PICK COLONY ON 1 PER CENT GLUCOSE HORMONE BOUILLON JUNE 4 TO JUNE 5					
D27H2	5.2	5.1	4.7	5.2	4.7	5.1	4.7	5.2	5.2	5.2		5.2		
D7H	4.6	4.6	4.8	4.6	5.0	4.5	4.8	4.6	4.6	4.6		4.6		
D14H	4.9	5.0	4.6	5.0	4.8	4.9	4.8	4.8	4.8	4.8		4.8		
D15H	5.0	5.0	4.7	4.9	4.7	5.0	4.6	5.1	5.0	5.0		5.0		
D16H	5.1	5.1	4.6	5.0	4.6	4.9	4.6	5.0	5.0	5.0		5.0		
D18H	4.8	4.9	4.5	5.0	4.7	5.0	4.6	5.1	5.0	5.0		5.0		
D22H	4.9	4.9	4.6	4.8	4.5	4.9	4.6	5.0	5.0	5.0		5.0		
D26H	5.3	5.3	4.8	5.4	4.7	5.2	4.8	5.4	5.4	5.4		5.4		
D25H	5.5	5.5	5.3	5.5	5.6	5.4	5.5	5.5	5.5	5.5		5.5		
D31H	5.3	5.3	4.8	5.2	4.8	5.2	4.9	5.3	5.3	5.3		5.3		
D32H	5.0	5.0	4.6	5.1	4.6	5.0	5.1	4.8	4.9	4.9		4.9		
A18H3	5.4	5.4	5.1	5.3	4.9	5.0	5.1	5.3	5.3	5.3		5.3		
A14H	5.3	5.3	4.9	5.3	5.4	5.0	5.4	5.3	5.3	5.3		5.3		
D24H	4.8	5.0	4.6	5.3	5.4	5.0	5.4	5.1	5.4	5.4		5.4		

Viridans

D4V2	5.1	5.1	4.6	5.0	4.8	5.1	4.7	5.1	4.6	Changed to hemolytic
D5V	5.3	5.2	4.6	5.0	5.0	4.6	4.6	5.2	4.7	
D6V	4.9	4.8	4.7	5.3	5.0	4.9	4.9	5.2	4.9	
D9V2	5.4	5.3	4.8	5.3	5.1	5.4	4.8	5.2	4.8	Changed to hemolytic
D11V	4.9	5.0	4.6	5.1	5.1	5.4	4.8	5.2	5.0	Changed to hemolytic
D12V2	4.7	4.6	4.6	5.0	5.0	5.3	4.8	4.7	5.2	Changed to hemolytic
D20V	5.0	4.9	4.9					4.8	4.7	Changed to hemolytic
D1V	5.1	5.0	4.6	5.4	5.0	5.1	4.7	5.0	4.8	
A16V	4.7	4.6	4.3	4.6	4.6	4.7	5.0	4.7	4.7	
A9V	4.8	4.8	4.7			4.8	5.1	5.0	5.0	Changed to hemolytic

same strain kept on Holman's cooked meat medium for thirty days without transplanting, the environment of this same strain on the two media differs considerably. Holman's meat medium is an excellent medium for anaerobes as well as aerobes. It is used in this laboratory for preserving stock cultures of anaerobes.

TABLE 3
Scalp wound, 2 days after injury

NUMBER OF STRAINS	FERMENTATION OF			LIMITING H-ION CONCENTRATION
	Lactose	Mannitol	Salicin	
Hemolytic				pH
4	+	-	+	5.2 to 5.4
4	+	-	+	4.3 to 4.5

Same wound, six days later, clinically a severe streptococcus infection

Hemolytic				
6	+	-	+	5.3 to 5.5
Viridans				
2	+	-	+	5.4
2	-	-	-	5.4

Toe wound, two days after injury

Hemolytic				
4	+	-	+	5.0 to 5.5
1	+	-	-	5.3
3	+	-	-	4.2 to 4.5

Same wound, four days later, clinically a severe streptococcus infection

Hemolytic				
14	+	-	+	5.0 to 5.5
1	+	-	-	5.3
1	+	-	+	4.3

The morphology of the streptococci, both hemolytic and non-hemolytic, changes on the Holman's cooked meat medium. The individual coccus becomes larger, the chains shorter, the gram stain is not so strongly positive; but these characteristics are lost after plating out on blood agar and growing for one generation in glucose bouillon.

Of the strains isolated from the throats of the diphtheria patients in this series, most of them fermented lactose and salicin, but not mannitol. We have called attention to the sugar fermentation characteristics of strains of streptococci isolated from wounds.

The strains of hemolytic streptococci that are most numerous in inflammatory lesions are the lactose and salicin fermenting types, the *Streptococcus pyogenes* (Holman 1916). This type seem to increase in number more than the other sugar fermenting types. Accompanying this change in the fermentation characteristics of the streptococcus flora in an inflammatory exudate is a change in the limiting hydrogen-ion concentration from pH 4.2 to 4.5 to pH 5.0 to 5.5. We have many strains of streptococci isolated from various sources, that have been on Holman's meat medium, with occasional transplantation, for two years. The sugar fermentation of these strains has not changed during this time. Under these conditions of artificial culture the strains are constant in their sugar fermentation characteristics. Whether there is a change in the sugar fermentation of the strains in an inflammatory area leading to a predominance of the lactose and salicin fermenting types, or whether this particular strain finds in such an environment an ideal culture medium for growth, remains an open question.

The limiting hydrogen-ion concentration is more variable than the sugar fermentation characteristics of the streptococcus. If the limiting hydrogen-ion concentration of a strain, either hemolytic or non-hemolytic, is between pH 4.8 to 5.8, it is probably to be interpreted as indicating that that particular strain has been present in an area where it has come in contact with inflammatory exudates. Further work is in progress as to the pathogenicity for mice of strains, with varying hydrogen-ion concentrations, isolated from wounds of patients with a streptococcus infection.

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COAGULATION AND STERILIZATION OF LOEFFLER'S BLOOD SERUM MEDIA UNDER STEAM PRESSURE

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The coagulation and sterilization of Loeffler's blood serum media has always involved a rather complicated technic with the results usually unsatisfactory in the end. Even with the use of the most modern inspissator, a pure ivory white media free from bubbles and irregularities, and in the end assured of sterility, is seldom obtained outside of the large biological laboratories. The difficulty that has always been encountered is that perfect slants cannot be obtained, through the exposure of the media to too rapid a process of heating or to too high a degree of heat.

This difficulty, however, is entirely overcome by a process of coagulating the media in completely sealed and air tight tubes. In this manner the expansion of the air, and consequent rise of pressure within the tubes individually, will prevent any formation of bubbles or irregularities of slants through expansion of the media. With the media in the tubes thus confined and held in a rigid position, 10 pounds of pressure may be applied without producing any shrinkage of the slants. The coagulation, however, is firm and the color of the media a pure white, with the surface entirely smooth. By employing a little care in the collecting of the blood and thus preventing extensive contamination with highly resistant spore bearing organisms, the whole process of coagulation and sterilization may be carried out in less than thirty minutes. The writer has employed the following process with complete success and satisfaction:

1. A sufficient amount of blood is collected in a sterile container so that the desired amount of serum can be obtained within two or three hours after it has clotted. As soon as possible after

collecting, the blood is set in a refrigerator to prevent the multiplication of such contaminating organisms as may have entered in the process of collecting.

2. After the desired amount of serum has separated from the clot, it is poured off into a sterile flask and centrifuged so as to remove such corpuscles as have been carried out by the serum.

3. The proper proportions of serum and glucose broth is now made up and the media distributed in sterile tubes of uniform length and firmly corked with a no. 1 grade of corks.

4. After this the tubes are placed in a metal rack, as shown in the accompanying illustration (fig. 1) and the cover screwed

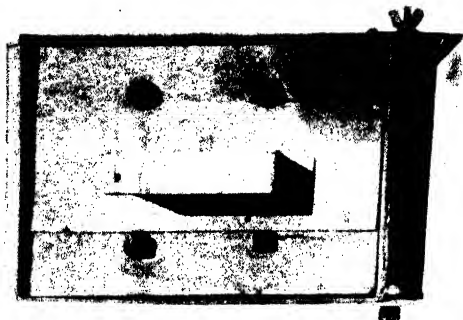


FIG. 1.

down firmly on the corks so as to prevent them from blowing out when the air within the tubes begins to expand as a result of heating.

5. The rack is so constructed that it may be fixed in a tilting position, giving to the tubes any desired slant. After the tubes have been properly packed into the rack and the cover firmly screwed on, the rack is set into the autoclave and the pressure allowed to rise as rapidly as it will under the ordinary amount of heat employed up to 10 pounds, when the heat is turned off and the indicator of the pressure gauge allowed to go back to 0.

6. After the pressure has entirely gone down within the autoclave, the cover is removed and the rack set out in the open to complete the cooling process, which takes some time. When it is certain that the corks will no longer blow out when the cover

of the box is removed, it may be taken off and the process of coagulation and sterilization will be found complete. The media will be of the best quality that can be produced, both in appearance and in actual culture work. An occasional tube may be found at times that still contains an organism that has not been killed. If too much contamination at the time of collecting is suspected, the tubes may be left in the rack with the cover screwed on until the next day when the medium is given another sterilization, the same as on the preceding day.

THE EFFECT OF CERTAIN BACTERIA UPON THE TOXIN PRODUCTION OF *BACILLUS* *BOTULINUS* IN VITRO

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Doubtless one of the outstanding problems of anaerobic bacteriology relates to the distribution of obligate anaerobes in nature. In August, 1920, we began a study of the distribution of the toxin forming anaerobes, by testing the toxicity of glucose broth filtrates from impure cultures. Failing to detect any toxin in the cultural filtrates from 24 specimens of soil and 10 of human feces, we were led to investigate, first, the methods suitable for such determinations, and second, the effect of microbic association upon toxin production by *B. botulinus*.

THE DISTRIBUTION OF *B. BOTULINUS* IN NATURE

Van Ermengem (1897) was the first to isolate *B. botulinus* from poisonous food, and its frequent recovery since from a wide variety of food products raises the question of its origin in them. The first record of *B. botulinus* from a source other than food or victim is that of Kempner and Pollack (1897), who came to believe that this organism sometimes inhabits the intestines of healthy swine, which explained the fact that botulism was then chiefly associated with pork products, particularly sausages.

Dickson (cited by Burke, 1919) failed to demonstrate botulinus toxin either in the intestinal contents of 250 grain-fed hogs from the abattoirs of South San Francisco, California, or in 10

¹ Aided by grants from the National Canners Association to the Hooper Foundation for Medical Research, and from the Board of Research at the University of California in 1920-21 and 1921-22.

samples of manure from 2 separate groups of garbage-fed hogs. Hence, it seems unlikely that this organism is a normal intestinal inhabitant in hogs of this region; but the results about to be reported justify further examinations.

During the summer of 1918, Burke (1919) obtained botulinus toxins from 2 out of 9 mixed cultures in glucose broth of bruised and bird-pecked cherries, from 1 out of 5 of hog manure, from 1 out of 3 of pole string beans spotted by insects, and from 1 of a small spider—collected from 5 localities in California, at 4 of which botulism had occurred during the previous winter.

Meyer and Geiger (1921) demonstrated botulinus spores upon market vegetables in San Francisco, and in two manured soils where canned beans had been grown and later caused limberneck in chickens. Since then Dr. Meyer² has shown an exceedingly wide distribution of *B. botulinus* in soils.

PROCEDURE FOR CULTURING FECES AND SOILS

Of the soil samples, 1 gram was weighed aseptically and transferred to a constricted tube (Hall, 1915) containing 20 cc. of 1 per cent glucose broth previously boiled five to ten minutes to drive out oxygen. Of the fecal samples, 1 cc., if liquid, or a lump the size of a pea, if solid, was used for inoculation. By gently rolling the tube, as much of the sample as possible was manipulated under the marble seal.

The cultures were incubated at 37°C. for seven to eleven days. They invariably showed abundant growth as indicated by gas and turbidity and were then filtered through sterile Berkfeld filters. The filtrates were preserved on ice to prevent deterioration.

A sterility test of each filtrate was made immediately after filtration by adding 1 cc. to a constricted tube of glucose broth. If no growth occurred during four days' incubation at 37°C., the filtrate was regarded as sterile. In a few instances contaminations developed because of defective filters; these filtrates were refiltered before testing for toxicity.

² Personal communication.

Toxicity tests were made by subcutaneous injection of guinea pigs. But, as stated in the beginning, none of the filtrates from 10 specimens of feces and 24 of soil was toxic. And since *B. tetani* and *B. botulinus* were well known to produce strongly toxic filtrates in pure glucose broth cultures in the constricted tube the failure to detect toxin in cultural filtrates from so many soils, some at least of which, being fertile, might be presumed to contain *B. tetani*, suggested some defect in our method. The distribution problem was therefore abandoned temporarily in order to determine what the factors of error were by testing for tetanus and botulinus toxins in filtrates of soil cultures inoculated with known strains. This paper presents the experiments upon *B. botulinus* only.

PURE AND IMPURE SOIL CULTURES OF *B. BOTULINUS* IN VARIOUS MEDIAS AND CONTAINERS

In the following experiments, the procedures already described for culturing samples and securing filtrates were used unless otherwise stated. Our *B. botulinus* 8A, (see Hall, 1922) was used.

Of the toxic filtrates an approximate determination of the minimum lethal dose was made. Each guinea pig was given an initial subcutaneous injection of 1 cc. of filtrate diluted 1:1000. If no symptoms had developed by the fourth day, the dose was increased 10 times, and if this proved to be non-toxic it was increased 10 times again. Long experience in immunizing animals to the toxins of tetanus and botulism teaches that no appreciable error is involved in making rough determinations of toxicity by this method, and it has the advantage of economy in animals. It was not possible always to use guinea pigs of constant weight, but a careful record of the weights was kept, in order to enable us to account for discrepancies attributable to this factor.

Autopsies were made of all guinea pigs that died. Heart blood cultures were planted in constricted tubes of glucose broth, and cultures were made of all visible lesions. No deaths could be attributed to bacterial infections, except in a few instances where

death resulted from a streptococcus epizootic among the guinea pig stock. In these cases, new guinea pigs were injected with the same dose of filtrate; only those animals dying specifically from botulism alone were counted.

THE EFFECT OF GLUCOSE IN THE MEDIUM. SOIL
CULTURES OF *B. BOTULINUS* IN GLUCOSE
BROTH AND PLAIN BROTH

The media for this series of experiments were 1 glucose broth and plain broth adjusted by adding an excess of MgCO_3 , boiling, and filtering out the undissolved residue. The reaction of such a medium is about pH 8.4. The following diverse soils were chosen.

Soil No. 9—clay—Contra Costa County, California.

Soil No. 12—sandy loam—Tulare County, California.

Soil No. 13—gravelly adobe—Tulare County, California.

Soil No. 14—gravelly loam—Glen County, California.

Each soil was cultured in plain and glucose broth in constricted tubes and inoculated with a large loopful of a deep brain culture of *B. botulinus*. Pure cultures of *B. botulinus* No. 8A in plain and glucose broths controlled each set. After incubating the cultures at 37°C. for eight to eleven days, their filtrates were tested for toxicity with the results shown in table 1.

It is clear from the table that in glucose broth there was either inhibition of growth, inhibition of toxin production, or destruction of toxin in the soil cultures. There was no clear-cut evidence either for or against inhibition of growth of *B. botulinus*; one would assume that growth of an anaerobe in the presence of aerobes and of the added particles of soil would be enhanced. The results seemed due rather to inhibition of toxin production or to toxin destruction.

In plain broth the pure control filtrate was toxic only in a dose of 1 cc.; curiously also were 2 of the soil filtrates, but the other 2 killed in doses of 0.01 cc. *B. botulinus* in pure culture grows poorly in mediums without fermentable carbohydrates and rarely produces strong toxin, but the above experiment and

others yet to be described show that under certain conditions of association with aerobes in plain broth stronger toxin is produced than in pure cultures.

TABLE 1
The effect of glucose in soil cultures of B. botulinus

Plain broth

SOIL SAMPLE	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
	grams	cc.	grams	cc.
9	540	1.0	—	—
12	560	1.0	—	—
13	280	0.001	460	0.01 (10)
14	340	0.001	410	0.01 (5)
Control (pure)	600	0.1	600	1.0 (4)

Date of culturing 10/1/20—incubation period 11 days.

() with number indicates days necessary to kill.

Some soil cultures in plain broth with *B. botulinus* are non-toxic; others are more toxic than the pure control.

Glucose broth

SOIL SAMPLE	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
	grams	cc.	grams	cc.
9	290	0.01	450	0.1 (4)
12	370	1.0	—	—
13	220	0.01	430	0.1 (3)
14	260	1.0	—	—
Control (pure)	330	0.0001	440	0.002 (6)

Date of culturing 9/28/20—incubation period eight days.

() with number indicates days necessary to kill.

Soil cultures in glucose broth with *B. botulinus* are either non-toxic or much less toxic than the pure control.

THE TIME FACTOR IN PRODUCTION OF BOTULINUS TOXIN

Considering the unavoidable difference in incubation periods in the above experiment, we next tested the effect of varying incubation periods upon the strength of the toxin, secured from pure cultures.

Four constricted tubes of plain broth and four of 1 per cent glucose broth were inoculated with a large loopful of a deep

brain culture of *B. botulinus*. One tube of each series was removed after each three day period of incubation, filtered and tested for toxicity, with the results shown in table 2.

None of the filtrates in the plain broth series was more than weakly toxic. The three day culture was the most active and there was a gradual decrease in toxicity in the six and nine day cultures, while the filtrate of the twelve day sample was non-toxic in a dose of 1 cc. With glucose broth there was a gradual increase in toxicity up to the twelfth day.

TABLE 2
Time factor in production of botulinus toxin by pure cultures

INCUBATION PERIOD	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
Plain broth				
<i>days</i>	<i>grams</i>	<i>cc.</i>	<i>grams</i>	<i>cc.</i>
3	500	0.1	170	1.0 (2)
6	360	0.1	300	1.0 (3)
9	400	0.1	400	1.0 (4)
12	330	1.0	—	—
Glucose broth				
3	430	0.001	490	0.01 (9)
6	—	—	300	0.01 (4)
9	—	—	460	0.001 (6)
12	—	—	530	0.001 (7)

Date of culturing 11/6/20.

() with number indicates days necessary to kill.

Only weak toxins were found in plain broth, and the strength decreased slightly with prolonged incubation; much stronger toxins were formed in glucose broth, and the strength increased at 37°C. up to nine days of incubation.

It is clear that glucose is favorable to toxin production in pure cultures of *B. botulinus* and that the time factor was not responsible for the differences between the glucose broth and plain broth filtrates of the pure cultures in our first experiment.

We then took an inventory of methods for detecting botulinus toxin in mixed cultures. Burke (1919) had used glucose broth under oil; we had been unsuccessful, or at least only partially successful, in using glucose broth in constricted tubes with soil

cultures heavily seeded with *B. botulinus*, notwithstanding pure cultures produced strong toxin under these conditions. Dr. K. F. Meyer told us that he was having success in the use of a 0.5 per cent glucose broth medium containing chopped meat in a strong bottle of about 100 cc. capacity fitted with a rubber stopper carrying a cotton plugged glass tube for connection with a suction pump. After evacuating for about fifteen minutes the tube was hermetically sealed in the flame. This method differed from the one that we had tentatively adopted, not only in providing a medium of higher buffer capacity in the chopped meat, but also in the exclusion of obligate aerobic bacteria.

We decided to determine which of these factors was responsible for our failure.

USE OF DIFFERENT MEDIA IN THE VACUUM BOTTLE

Comparative studies were made on *B. botulinus* in 1 per cent glucose broth and 0.5 per cent glucose meat mash medium with and without soil no. 9, which was used in these tests in the bottles. There were about 50 cc. of medium in each bottle, and half of the volume was meat in the chopped meat medium. The cultures were incubated at 37°C. for one week. Both the pure and soil cultures of *B. botulinus* in the meat mash medium proved very toxic, the minimum lethal dose of each filtrate being 0.001 cc. The filtrate from the soil culture in glucose broth was also toxic, with a minimum lethal dose of 0.01 cc. indicating that the inhibition of obligate aerobes might be one factor in the success of this method. No growth occurred in the pure glucose broth culture, owing possibly to imperfect anaerobiosis.

USE OF DIFFERENT MEDIA IN THE CONSTRICTED TUBE

A similar experiment was conducted using the constricted tube with plain broth and plain meat mash medium. The tubes were boiled before inoculating.

As shown in table 3 the filtrates were non-toxic in the pure and only weakly toxic in the soil broth cultures. However, in the meat mash medium, the filtrates from both pure and soil

cultures were very toxic, 1 cc. of a 1:10,000 dilution killing guinea pigs in three days.

These experiments caused us to conclude that the inhibition of obligate aerobes in the vacuum bottle is not the only factor in the success of this method; the buffer nature of the meat mash medium plays perhaps an even more important rôle, for the constricted tube which proved equally successful when used with this medium does not inhibit aerobic growth above the seal. *Botulinus* toxin can be detected in filtrates from mixed soil cultures in a meat mash medium with the constricted tube,

TABLE 3

Comparative studies on meat digest medium and plain broth in the constricted tube in pure and soil cultures of B. botulinus

CULTURE	MEDIUM	WEIGHT OF GUINEA PIG	FATAL DOSE
		grams	cc.
Pure <i>B. botulinus</i>	Plain broth	—	—*
Soil 9 + <i>B. botulinus</i>	Plain broth	440	0.1 (3)
Pure <i>B. botulinus</i>	Meat mash	260	0.0001 (3)
Soil 9 + <i>B. botulinus</i>	Meat mash	250	0.0001 (3)

* 1 cc. failed to kill a guinea pig of 790 grams weight.

() with number indicates days necessary to kill.

Note the great toxicity of the filtrates from meat mash medium.

and the earlier failure to detect it in filtrates from cultures in glucose broth cannot be blamed upon the type of container used, but rather upon the medium, which fails under the peculiar conditions of the test, although pure cultures of *B. botulinus* produce strong toxin in it.

THE EFFECT OF GLUCOSE IN THE MEAT MASH MEDIUM

We then undertook to determine the difference, if any, due to glucose in meat mash in constricted tubes with pure and soil cultures of *B. botulinus*, also, to see if the absence of the marble seal affected toxin production in either pure or soil cultures.

The meat mash medium was made according to the following formula:

- (a) 1 pound of ground meat per liter of water
- (b) 0.5 per cent NaCl
- (c) 2 per cent Bacto-peptone
- (d) Adjusted to pH 7.2 with NaOH
- (e) Sterilized in the Arnold sterilizer on three successive days

TABLE 4

Effect of glucose in a meat mash medium in the constricted tube

CULTURE	MEDIUM	MARBLE SEAL	WEIGHT OF GUINEA PIG	FATAL DOSE
			grams	cc.
<i>B. botulinus</i> 8A	Plain meat mash	Present	600	0.001 (4)
	1 per cent glucose meat mash	Present	650	0.001 (4)
	Plain meat mash	Absent	720	0.01* (3)
	1 per cent glucose meat mash	Absent	550	0.001 (10)
<i>B. botulinus</i> plus soil 9	Plain meat mash	Present	610	0.0001† (65)
	1 per cent glucose meat mash	Present	350	0.0001 (5)
	Plain meat mash	Absent	640	0.001 (6)
	1 per cent glucose meat mash	Absent	690	0.01 (2)‡

Date of culturing 8/25/21—incubated at 37°C., seven days, on ice one week.

* 0.001 cc. had failed to cause symptoms in this guinea pig.

† Very sick with chronic botulism for two months before death.

‡ No more filtrate to test.

Eight constricted tubes were filled with 20 cc. of this medium, with or without 1 per cent glucose, added as indicated, the ground meat occupying about one-half of the space occupied by the liquid in each tube. Four tubes were inoculated with 1 gram of soil 9, as shown in table 4, and all with *B. botulinus*. After incubating at 37°C. for seven days, the cultures were kept on ice for one week before filtering, awaiting the arrival of filters.

Stronger toxins were obtained with than without marble seal in both pure and soil cultures of *B. botulinus* 8A in plain and 1 per cent glucose meat mash medium, owing perhaps to the

more strictly anaerobic conditions with the seal. It was apparent, however that the ground meat helped to exclude oxygen since no growth of anaerobes in pure culture occurred in constricted tubes without seals in clear media unless the depth were increased greatly.

With the marble seal the factor of added glucose in soil cultures in the meat mash medium could be disregarded; equally strong toxins were produced in its absence. Of course the meat contained some muscle sugar. With pure cultures of *B. botulinus* without the seal there was a slightly stronger toxin in 1 per cent glucose meat mash than in plain meat mash.

Possible differences between plain and glucose soil cultures without seals could not be determined, owing to the slight quantity of the last filtrate available for testing. The soil cultures in both plain and glucose meat mash medium, with or without the marble, resulted in stronger toxins than in the corresponding pure cultures.

These experiments proved that the inhibitive factor in our earlier attempts to demonstrate strong botulinus toxin from soil cultures in constricted tubes of glucose broth might be overcome through the use of more heavily buffered mediums. In fact, either plain or glucose meat mash, with or without the marble seal, can be used for detection of *B. botulinus* in soil samples.

STERILE SOIL CULTURES OF *B. BOTULINUS*

Dr. C. B. Lipman of the Soil Chemistry Department of this university suggested that soils might inhibit the toxin production of *B. botulinus* by chemical action.

Samples 9, 12, 13, and 14 were sterilized in the autoclave for one hour at 18 pounds pressure. One gram amounts of each were placed below the marble in constricted tubes of 1 per cent glucose broth. These tubes were sterilized in the autoclave for one-half hour, after which they were incubated at 37°C. for forty-eight hours. Since no growth appeared during this period, they were regarded as sterile. Each soil culture was then

inoculated by loop from a deep brain culture of *B. botulinus*, along with a control in glucose broth without soil. The cultures were incubated eight to eleven days, and grew actively. They were then filtered and tested for toxicity, with the results shown in table 5.

It is evident that there may have been some slight inhibitive or destructive effect upon the toxin. All the soil cultures gave weaker filtrates than the control, but the sterile soil cultures were considerably stronger than any secured in glucose broth in constricted tubes with non-sterile soils. It is possible that inhibitive substances were destroyed or volatilized by heating.

TABLE 5
The effect of sterile soils in glucose broth cultures by B. botulinus

SOIL SAMPLE	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
	<i>grams</i>	<i>cc.</i>	<i>grams</i>	<i>cc.</i>
9		*	700	0.001 (7)
12	710	0.001	760	0.01 (3)
13		*	690	0.01 (3)
14	230	0.001	320	0.01 (4)
Control (pure)		*	690	0.001 (2)

Date of culturing 2/4/21—period of incubation eight to eleven days.

() with number indicates days necessary to kill.

* The non-fatal dose was not determined where the animals died on or after the third day; in such cases one-tenth the fatal dose rarely causes symptoms.

THE EFFECT OF SOIL AEROBES UPON TOXIN PRODUCTION

Having shown that the chemistry of the soils apparently could not account wholly for the failures in our first experiments the problem of a possible bacterial antagonism was next considered.

Soils 9, 12, 13 and 14 were cultured in glucose broth. After twenty-four hours' incubation at 37°C. each was successively subcultured several times on agar slants to eliminate the anaerobes.

Gram stains showed mixtures of hay bacilli, staphylococci and Gram negative non-sporulating rods. Each mixed culture was inoculated in a constricted tube of glucose broth along with

B. botulinus 8A, with the usual pure control. The cultures were incubated for seven days, then filtered and tested for toxicity.

The pure control filtrate killed a guinea pig (weight 220 grams) in a dose of 0.001 cc. in three days with botulism. But while some of those (weights 300 to 370 grams) inoculated with 1 cc. of the filtrates from the mixed cultures showed definite symptoms of botulism, none died.

This experiment showed conclusively that the aerobic bacteria found in these four soils were able to prevent the accumulation of strong botulism toxin in the culture, in spite of the generally accepted view that admixture of aerobes with anaerobes favors the latter through the reduction of oxygen tension.

HISTORICAL REVIEW OF AEROBE-ANAEROBE SYMBIOSIS

Shortly after Pasteur (1861) discovered his "Vibrien butyrique," the first known obligate anaerobe, he recognized that in nature this and similar organisms must often depend upon the abstraction of oxygen from their environment by aerobic microorganisms in order to grow. This explanation was accepted by Roux (1887), who utilized the hay bacillus as a technical aid in the cultivation of *Vibrien septique*, by Penzo (1891), who cultivated the "bacillus of malignant oedema" symbiotically with *Erythrob. prodigiosus* and *Proteus* in broth, and by Novy (1894), who did the same thing with his *B. oedematis-maligni* II (B. Novyi), adding *Bact. acidi-lactici* and a coccus to the list of successful symbionts.

In opposition to Pasteur's explanation of the symbiotic growth of anaerobes with aerobes Kedrowsky (1895) formulated a "vital ferment" theory which, however, is generally regarded as disproved by the work of Scholtz (1898), Bienstock (1899) (1903), Matzuschita (1902), Von Oettingen (1903) and others.

The symbiotic development of anaerobes other than *B. botulinus* in the presence of various aerobes has been shown in the writings of Smith (1898) (1908), Rosenthal (1902), Proca (1907), Marino (1910), Francis (1914), Torrey (1917), McCoy and Bengtson (1918), Koser and McClelland (1918), Wilson and

Steer (1918), Sturges and Rettger (1919), Rhein (1919), and Adamson (1920).

While most of these deal simply with the phenomenon of growth, Smith's (1898) (1908) observations are of particular interest in showing the inadequacy of Arnold sterilization of media intended for the production of diphtheria toxin, and those of Francis (1914) and McCoy and Bengtson (1918) in showing the lack of toxicity in tetanus cultures grown in glucose broth with certain fermentative aerobic bacteria.

Certain recent writers have used symbiotic methods technically, as for example, Koser and McClelland (1918) in securing spore suspensions of *B. tetani* and *B. putrificus*, Wilson and Steer (1918), in making fermentation tests of anaerobes; and Sturgess and Rettger (1919), in isolating *B. putrificus*.

While Bienstock (1906) found that *Bact. coli* and *Bact. lactis-aerogenes* inhibited the growth of *B. putrificus* in sugar media, Sturges and Rettger (1919) found that *Bact. coli* enhanced the putrefactive properties of *B. putrificus*.

Van Ermengem (1897), recognized the probable symbiotic rôle of sarcinae and micrococci found along with the newly discovered cause of botulism in the pickled ham under investigation. He showed experimentally that putrefactive mixtures of blood, feces, or urine failed to destroy the toxicity of cultures and that broth cultures grown symbiotically with *Erythrob. prodigiosus*, *Proteus*, *Ps. fluorescens*, and *Bact. coli* were highly toxic. The symbiotic growth in cultures of *B. botulinus* with various aerobes was also studied by Scholtz (1898), Bienstock (1903) and Proca (1907), and Dickson and Burke (1918) pointed out that *B. botulinus* is usually associated with other spores, notably of *B. subtilis*, and that "production of toxin is not prevented by the presence of other bacteria." Shippen (1919) emphasized the possible importance of symbiosis in the natural growth of this organism as an explanation of certain obscure outbreaks of forage poisoning in horses and showed experimentally that mixed cultures in broth with yeasts or *M. aureus* are still toxic. *Bact. coli* was found to inhibit toxin formation in milk, however, and we note an analogy here to the finding of Francis with

B. tetani, a sugar (lactose) not being fermented by the anaerobe but fermented with acid production by the aerobic symbiont and the cultures appearing non-toxic. Graham and his collaborators (1919) (1921) also recognized the probability of aerobic symbiosis of *B. botulinus* in nature.

There is further a tacit implication in most of the published determinations of botulinus toxin in food materials that bacterial contamination does not interfere with toxin production.

ANTAGONISTIC EFFECT OF CERTAIN AEROBIC BACTERIA UPON TOXIN PRODUCTION BY *B. BOTULINUS*

Having shown that mixed aerobic soil cultures prevent botulinus cultures from becoming toxic in glucose broth we then prepared to test various individual species including those recovered from the soil samples.

At this time we took account of the fact that the brain cultures of *B. botulinus* used for seeding purposes were frequently quite toxic in themselves, so that if a sufficiently large inoculum were used a culture might be rendered toxic from the beginning. We therefore determined what dilution of a brain culture would give growth in the constricted tube with 1 per cent glucose broth by making a series of dilutions, 1:10, 1:100, 1:1000, etc. Ten constricted tubes were inoculated in serial order with 1 cc. amounts of these dilutions.

The tubes inoculated with the first 8 dilutions, i.e., including the one inoculated with 0.00000001 cc., were all turbid within twenty-four hours at 37°C., and the first 5 also produced gas. In forty-eight hours all ten tubes were turbid and gas bubbles had collected below the marble seals, showing that one ten-billionth cc. brain culture is sufficient to induce growth. None of the tubes proved to be contaminated aerobically, and a filtrate of the culture inoculated with 0.00001 cc. proved toxic for a small guinea pig in a dose of 1 cc. 1:100 dilution.

Assuming a toxicity in brain cultures not exceeding 10,000 minimum lethal dose per cubic centimeter, if only 0.001 cc. were used to inoculate 20 cc. of glucose broth, 1 cc. would contain

0.00005 cc. or less than a fatal dose for a guinea pig. Inoculations of the succeeding glucose broth cultures were therefore made with 1 cc. 1:1000 brain culture.

It was never possible in several trials to repeat these results with plain instead of glucose broth. Larger inocula are usually necessary to secure growth in plain broth.

TECHNIQUE

A mixed culture of each aerobe with *B. botulinus*, a pure culture of each aerobe, and a pure culture of *B. botulinus* comprised each test. The aerobes were mostly tested in sets of 2 to 4, so that a single pure culture of *B. botulinus* served as control for all of that particular set. Aerobic inoculations were made from agar slants just checked for purity and identity. After growth had appeared in all the tubes, each culture was spotted upon agar to determine if the aerobe grew, and if so to find if it were pure. The botulinus control was required to be free from aerobes in every case. After seven to eight days' incubation the cultures were filtered, the filtrates tested for sterility and then for toxicity on guinea pigs, as previously described.

While the tests were always made in paired sets of plain and glucose broth, only the latter failed to produce as strong toxin in the mixed cultures as in the pure controls. There was no evidence of toxin inhibition by the aerobes in plain broth, in fact the pure controls were usually less toxic than the mixed culture filtrate, though all were weak. Of course the filtrates from the pure aerobic controls were all non-toxic in a dose of 1 cc.

Lack of space prevents the presentation of detailed data of which analyses are given below—first of the results with glucose broth, and then of those with plain broth, for contrast.

RESULTS WITH GLUCOSE BROTH

Pure control filtrates fatal in doses of 0.001 cc., not in 0.0001 cc.

Mixed filtrates fatal in doses of 0.001 cc., not in 0.0001 cc.

Unidentified bacillus

B. mycoides (soil)

B. pseudotetanicus (soil)

Staphylococcus albus

B. subtilis no. 1

Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.

None

Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.

B. subtilis no. 2

Erythrob. prodigiosus

Mixed filtrates fatal in doses of 1 cc., not in 0.1 cc.

Bact. coli-communior (soil)

Bact. coli-communis

Bact. typhosum

Pure control filtrates fatal in doses of 0.01 cc., not in 0.001 cc.

Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.

Bact. mucosum-capsulatum

B. anthracis

Bact. alkaligenes

Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.

None

Mixed filtrates fatal in doses of 1 cc., not in 0.1 cc.

Bact. dysenteriae (Duval)

Bact. coli-communior (Lab.)

Mixed filtrates non-fatal in doses of 1 cc.

Bact. paratyphosum A

Bact. paratyphosum B

Bact. enteriditis

Bact. dysenteriae (Shiga)

Inspection proves that certain of the most actively fermentative members of the genus *Bacterium* were responsible for the less toxic filtrates of *B. botulinus*. The analogy of this result with that of Francis (1914) for *B. tetani* is emphasized.

Bact. coli-communior was the only one of the soil aerobes that was found to inhibit toxin production. At the time of isolation, which was about five months after the mixed aerobic tests were made, it was found present in soils 12 and 14. It may have been present in soils 9 and 13 at the time when the mixed soil aerobes were tested with *B. botulinus*, but may have died before an attempt was made to isolate them.

Since none of the sporulating aerobes was found to inhibit toxin production one might expect successfully to demonstrate botulinus toxin in mixed glucose broth cultures of imperfectly sterilized canned foods or other sources harboring the spores of *B. botulinus* to which sufficient heat had been applied to destroy vegetative forms before culturing. Any method selective for spores however would probably overlook certain strains of *B. botulinus*, such as our No. 8A and 78B, which do not sporulate readily and are easily killed by boiling.

RESULTS WITH PLAIN BROTH

Pure control filtrates fatal in doses of 0.01 cc., not 0.001 cc.

Mixed filtrates fatal in doses of 0.001 cc., not in 0.0001 cc.

B. mycoides (soil)

B. subtilis no. 1

Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.

Bact. coli-communior (soil)

Unidentified bacillus (soil)

Staph. albus

B. subtilis no. 2

Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.

B. pseudotetanicus (soil)

Pure control filtrates fatal in doses of 0.1 cc., not in 0.01 cc.

Mixed filtrates fatal in doses of 0.01 not in 0.001 cc.

Bact. coli-communis (Lab.)

Mixed filtrates fatal in doses of 0.1 cc. not in 0.01 cc.

Bact. coli-communior

Bact. typhosum

Pure control cultures failing to grow.

Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.

Bact. enteritidis

Bact. alkaligenes

B. anthracis

Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.

None

Mixed filtrates fatal in doses of 1 cc., not in 0.1 cc.

Bact. dysenteriae (Shiga)

Bact. dysenteriae (Duval)

THE EFFECT OF CERTAIN ACID PRODUCING SPECIES OF THE
GENUS BACTERIUM ON BOTULINUS TOXIN

An experiment was made to determine if formed toxin is destroyed by the inhibitory organisms. Two lots of 400 cc. each of toxin were prepared, one in broth containing 3 per cent (an excess), the other 0.3 per cent (less than excess) glucose. Ground meat in a depth of about one-half inch was added to act as buffer. The cultures were incubated at 37°C. for four days and then filtered. That 0.3 per cent glucose was really less than excess was indicated by blackening of the meat and a terminal reaction of alkalinity. Thus, this toxin corresponded to plain broth. That 3 per cent glucose was an excess was indicated by failure of the meat to blacken and acid reaction; after filtration it still contained presumably some glucose. The two lots of toxin were distributed in tubes and tested for sterility by incubation at 37°C. over night; they were then inoculated from agar slants of the selected aerobes, and, with a control of sterile toxin, incubated at 37°C. for three days, filtered and tested for toxicity. The data are summarized herewith.

RESULTS WITH FILTRATE FROM BROTH WITH LESS THAN
EXCESS GLUCOSE

Uninoculated filtrate fatal in dose of 0.1 cc., not in 0.01 cc.

Inoculated filtrates fatal in doses of 0.1 cc., not in 0.01 cc.

Bact. coli-communior (Lab.)

Bact. coli-communior (soil)

Bact. coli-communis

Bact. typhosum

Bact. dysenteriae (Duval)

Inoculated filtrates fatal in doses of 1 cc., not in 0.1 cc.

Bact. paratyphosum A

Bact. paratyphosum B

Bact. dysenteriae Shiga

Bact. enteritidis

There was really less toxin destruction by the last four species in the 0.3 per cent glucose filtrate than indicated above, for while

with a toxicity of 0.1 cc. in the uninoculated controls this amount of filtrate from these cultures failed to kill guinea pigs, it invariably produced severe symptoms, and doses of 1 cc. killed much more promptly than the control dose of 0.1 cc.

RESULTS WITH FILTRATE FROM BROTH WITH EXCESS GLUCOSE

Uninoculated filtrate fatal in doses of 0.01 cc., not in 0.001 cc.

Inoculated filtrates fatal in doses of 0.01 cc., not in 0.001 cc.

Bact. typhosum

Bact. paratyphosum A

Inoculated filtrate fatal in doses of 0.1 cc., not in 0.01 cc.

Bact. dysenteriae (Duval)

Bact. enteritidis

Inoculated filtrate fatal in doses of 1 cc., not in 0.1 cc.

Bact. coli-communior (Lab.)

Bact. coli-communior (soil)

Bact. coli-communis

Bact. paratyphosum B

Bact. dysenteriae Shiga

The most actively fermentative species were the ones that destroyed the toxin and in order to confirm this we tested the terminal hydrogen ion concentration of the following pure cultures in glucose broth after incubation at 37°C. for forty-eight hours with the following results:

	pH
<i>Bact. enteritidis</i>	4.2
<i>Bact. coli-communis</i>	4.4
<i>Bact. coli-communior</i>	4.4
<i>Bact. coli-communior</i> (soil strain).....	4.4
<i>Bact. typhosum</i>	4.5
<i>Bact. paratyphosum</i> A.....	4.6
<i>Bact. paratyphosum</i> B.....	4.6
<i>Bact. dysenteriae</i> Shiga.....	5.4
<i>Bact. dysenteriae</i> Duvall.....	5.4
<i>Erythrob. prodigiosus</i>	5.6
<i>B. mucosum capsulatum</i>	5.6
<i>Staphylococcus albus</i>	5.8
<i>B. anthracis</i>	6.0
<i>B. mycoides</i>	6.0
Unidentified sporulating bacillus.....	6.2

<i>B. subtilis</i> no. 1.....	7.0
<i>B. subtilis</i> no. 2.....	7.2
<i>B. alkaligenes</i>	8.0
Broth.....	7.2

That acid production (pH 5.4 or less) by the aerobes was responsible for the destruction of toxin by *B. botulinus* as fast as it was formed in mixed cultures seems an inevitable conclusion. But it is well known that botulinus toxin differs from all other bacterial toxins in its tolerance for the gastric acidity, which ranges from pH 1 to pH 2. Bronfenbrenner and Schlesinger (1921, 1922) have shown that an acidity equal to that of the stomach actually increases the potency of botulinus toxin.

We performed the following experiment:

Botulinus toxin containing 1000 minimum lethal doses per cubic centimeter was diluted 1:10 in sterile distilled water to reduce its buffer content. 0.5 cc. of this dilution was then added to 4.5 cc. of buffer solutions prepared according to the formulae of Clark and Lubs (1917) with pH values of 1, 2, 4, 5, 6, 8, and 9.4 giving a 1:100 dilution of toxin in each tube. A comparison of the buffers containing toxin with undiluted buffers by the colorimetric method showed little or no change in reaction. They were then incubated at 37°C. overnight and 1 cc. (10 minimum lethal doses?) volumes of each injected into guinea pigs. The results were as follows:

pH	WEIGHT OF GUINEA PIG	RESULT
	grams	
1.0	150	Lived
2.0	160	Dead 18 hours
4.0	160	Dead 18 hours
5.0	180	Dead 18 hours
6.0	190	Dead 18 hours
8.0	180	Dead 6 days
9.4	160	Lived

We cannot claim from these results to have confirmed the findings of Bronfenbrenner and Schlesinger, but at any rate a pH value of 2.0 failed to destroy the toxin in the time allowed. It might be that a longer exposure comparable to what our

mixed cultures had would give a different result. The destructive action of alkali (pH 8.0 and pH 9.0) and strong acid (pH 1.0) was clearly evident.

Further experiments in line with our last one should be undertaken, for if acidities of pH 2 to 5 do not destroy botulinus toxin we shall be forced to conclude that certain fermentative aerobes destroy botulinus toxin through the operation of some mechanism involving a factor other than acid but dependent upon it.

SUMMARY

1. Toxin forming anaerobes could not be detected in soils or human feces by toxicity test of filtrates from glucose broth cultures in the constricted tube without pre-heating.

2. *B. botulinus* could not be detected by toxicity tests of filtrates from artificially inoculated soil cultures in glucose broth without pre-heating. Moderately toxic filtrates were not infrequently obtained from such cultures in plain broth, indicating a symbiotic relationship between *B. botulinus* and the bacterial flora in plain broth.

3. Meat mash medium was found to be very favorable for pure and artificially inoculated soil cultures of *B. botulinus* in both the vacuum bottle, and the constricted tube. The inhibition of aerobes in the vacuum bottle, although of some importance, is not the only factor involved in its success. The success of the meat mash medium may be attributed mainly to the high buffer content of the medium.

4. The constricted tube with marble seal was used successfully for the detection of *B. botulinus* in artificially inoculated soil cultures both in plain and in glucose meat mash medium.

5. Sterile soils did not inhibit toxin production by *B. botulinus*.

6. Impure aerobic cultures from certain soils were found to inhibit toxin production of *B. botulinus* in glucose broth.

7. Certain acid producing aerobes inhibited toxin production by *B. botulinus* in glucose broth; no inhibition occurred in plain broth.

8. Some of these acid producing organisms destroyed botulinus toxin in glucose toxin cultures.

9. Those organisms that gave non-toxic or very weakly toxic filtrates with *B. botulinus* produced the highest acidities in glucose broth.

10. A single experiment with buffer solution showed that an acidity of pH 2.0 did not injure the potency of botulinus toxin in 24 hours at 37°C.

11. The evidence points to the operation of a factor or factors other than acidity responsible for the inhibition of toxin formation or toxin destruction in mixed cultures of *B. botulinus* with acid forming aerobes.

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ON NITRIFICATION

V. THE MECHANISM OF AMMONIA OXIDATION

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INTRODUCTION

In a previous communication, on the subject of nitrification, the conclusion was reached that the oxidation of ammonia to nitrites takes place in two steps; a—one of respiration with resultant gain in energy and synchronous nitrogen absorption, b—the other of nitrogen assimilation, nitrification proper, whereby oxidation of the absorbed nitrogen takes place, the utilized portion going to make up the following cell generations, nitrous acid is split off and excreted as a non-utilizable product and energy is liberated. The phenomenon was given a diagrammatic representation.

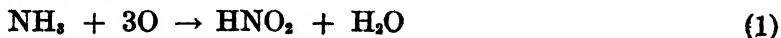
It is the object of the present paper to study the mechanism whereby these steps take place and contribute to our knowledge of the processes of chemosynthesis occurring in Nature.

The organism used throughout these studies was an active form of Nitrosococcus isolated from Wooster soils by the methods described elsewhere (Bonazzi, 1919) and kept in Omeliansky solution for over two years without rejuvenation in soils.

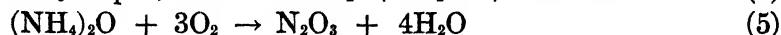
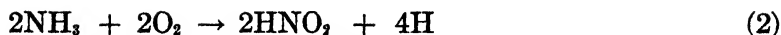
GASEOUS EXCHANGES DURING NITROSOFERMENTATION

General

In 1895 Godlewsky symbolized the process of nitrosofermentation by means of equation 1.



This same process has been symbolized by various authors as follows:



It is noteworthy that none of these equations is based upon a rigid experimental proof. It seems therefore that some attention should be given to the gaseous exchanges in active cultures of *Nitrosococcus*. As a matter of fact, existing data on the subject

TABLE 1

(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
SOURCE OF DATA	NUMBER OF CULTURE AND MEDIUM USED	NITROGEN CHANGES IN MILLIGRAM OF N			OXYGEN CONSUMED IN		R = $\frac{\text{O}}{\text{N}}$
		NH ₃ trans- formed	N ₂ O ₃ formed	N ₂ O ₅	Cubic centi- meter	Milli- gram	
Schloesing, 1885A.....	1 soil	159.40		155.80		787.0	4.42
	2 soil	112.30		109.70		552.0	4.40
	3 soil	84.60		82.78		467.0	4.90
1885B.....	1 soil	208.70		198.90		1280.0	5.63
	2 soil	362.80		299.70		1886.0	5.51
Godlewsky, 1892.....	1 solution		22.37		78.14	111.96	4.38
	2 solution		35.86		116.25	165.10	4.03
1895*.....	1 solution		48.40		117.03	166.20	3.00
	2 solution		49.00		119.23	169.30	3.03

* The values given by Godlewsky in 1895 and here reproduced, were obtained by use of cultural systems in which the gaseous phase contained at the start 3.8 and 3.5 per cent of CO₂ respectively.

are extremely meagre and contradictory as may be seen at a glance from table 1 where values obtained by two observers are given for the ratio $R = \frac{\text{O}}{\text{N}}$ where O is the quantity of oxygen absorbed by the culture, expressed in gram atoms, and N is the quantity of nitrogen nitrified also expressed in gram atoms.

The discrepancies between the data presented by the two authors may well be explained on the basis of the fact that the work of Schloesing was done with soil and that in this medium

oxidations are always going on besides the ones involved in the actual process of nitrification proper. This consideration would naturally call for a greater consumption of oxygen than could be accounted for on the basis of the nitrogen nitrified. The fact also that the ratios obtained are the same for the same soil whereas they differ in the various soil types (the values of 1885a versus those of 1885b) is sufficient to point to the oxidation of some component inherent to the soil makeup.

The values obtained by Godlewsky (in solution cultures) are somewhat perplexing, but it should be here emphasized that the cultures used by this author were crude, and only in their second transfer from soil. Again, it should also be mentioned that the data were not obtained by Schloesing and by Godlewsky with the apparent purpose of studying the mechanism of ammonia oxidation. However, since they are the only values given in the literature on the gaseous exchanges in nitrification they are here tabulated to indicate that no definite concordance of existing data justifies the establishment of an equation for the phenomenon of nitrosofermentation. In this connection it should be mentioned that Meyerhoff in all his work on the respiration of the nitrous organism uses the theoretical value of $R=3$ and calculates by means of this factor the quantity of nitrogen nitrified from the quantity of oxygen consumed.

In view of the above considerations and in the hope of reaching a better understanding of the gaseous exchanges during nitrosofermentation the following series of experiments was undertaken.

Experimental

The ordinary Omeliansky solution with excess of $MgCO_3$ was used except for the introduction of ammonium carbonate in some cases in place of the sulphate. Naturally in such cases the magnesium carbonate was omitted from the solutions. The flasks with ground-glass-jointed manometers or glass flame-sealed joints, were similar in principle to those described in another publication (Bonazzi, 1921). As has been stated above, the organism used was isolated from Wooster soil.

The gaseous exchanges were studied in some cases by transferring to the special gas flasks, by means of sterile pipettes, large quantities (25 or 50 cc.) of cultures in intensive nitroso-fermentation, replenishing the supply of ammonium sulphate, and sealing the flasks after obtaining samples of the gas from the system.

In other cases (cultures 331-1 and 331-2 of table 2) the sterile solution contained in the gas flasks was inoculated with a loopful of an active culture and the system closed after obtaining a sample of the gas for analysis. After varying periods of incubation, the flasks were evacuated by means of a mercury pump, the

TABLE 2

(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)	(ix)
NUMBER OF EXPERIMENT	DAYS OF INCUBATION	OXYGEN IN AIR			NITROGEN AS NITRITE			RATIO $R = \frac{O}{N}$
		At start	At end	Lost	At start	At end	Formed	
		cc.	cc.	mgm.	mgm.	mgm.	mgm.	
271-1	14	17.14	4.06	18.68	1.68	7.47	5.79	2.82
4	14	16.79	5.16	16.62	1.68	7.17	5.49	2.71
328-1	7	16.93	6.37	15.09	9.03	13.64	4.61	2.87
7	7	16.37	4.43	17.06	9.15	14.28	5.13	2.91
6	8	16.82	5.17	16.64	8.73	13.79	5.06	2.88
3	11	17.28	4.27	18.59	9.11	14.38	5.27	3.09
4	11	17.31	1.95	21.94	8.34	14.59	6.25	3.07
2	17	16.97	5.08	16.98	9.14	14.14	5.00	2.97
331-1	50	16.34	10.52	8.31	0.00	2.66	2.66	2.73
2	22	17.10	1.61	22.13	0.16	6.79	6.62	2.92

gases collected over mercury, measured and used for analysis. In all this work ammonia and nitrites were determined by the distillation, and reduction and distillation methods developed in this laboratory (Allen and Davisson).

The results of a number of experiments are summarized in table 2, where R is again calculated as was done for table 1.

Culture 331-1 received ammonium carbonate instead of the sulphate. The average value of R is found to be 2.89 ± 0.08 , ranging between 2.81 and 2.97 when the error of the various determinations is calculated according to the method of least

squares. Within the limits of error the value of R is therefore ± 3 indicating that for every atom of nitrogen nitrified three atoms of oxygen are consumed by the organisms, from the surrounding atmosphere.

This conclusion conforms to the generally accepted theory, and offers justification of equations 1, 3, 4 and 5 given above.

IODINE AND NITROSOCOCCUS

General

Müntz in 1885 ascribed to the "organisme qui effectue la nitrification des matières azotées" the power of oxidizing the iodine of iodides to iodates. However, an examination of his experimental procedure shows that percolation of a nitrifiable solution, to which iodides have been added, through gravel inoculated with compost and the subsequent detection of iodic acid in the percolate is not sufficient proof to justify the conclusion that the organisms active in the oxidation of the iodides are the nitrifying organisms. Müntz's investigations were carried out long before the actual isolation of the two groups of oxidizing organisms concerned with the nitrogen cycle, so that it is not surprising if this author reached the conclusion mentioned above. Müntz explained the presence of the iodates in the Chile deposits on the assumption that the nitrifying organisms played an important rôle in their production.

From a theoretical standpoint proof of this hypothesis would be of great importance and accordingly experiments were undertaken with the object of studying the question further.

Experimental

The first step in the study was to find whether any retarding effect was exerted by iodides on the process of nitrification. Boullanger and Massoll (1903-1904) found that ammonium iodide was easily nitrified when the concentration of this salt was such that the ammonium ion was in a dilution equal to that which it attains in the ordinary Omeliansky solution.

A series of flasks, each containing 25 cc. of a culture of *Nitrosococcus* from Wooster soil in full nitrification, and which had nitrified 0.6 grams of ammonium sulphate, were used. After renewal of the ammonia supply in the form of 0.5 cc. of a 10 per cent sterile solution of ammonium sulphate, some of the cultures received 0.5 cc. of a sterile one per cent solution of potassium iodide. Tests for ammonia were made on successive dates and it was found as was to be expected from the work of Boullanger and Massoll, that no deleterious influence was exerted by the iodide upon the process of ammonia oxidation.

The next point for investigation was the actual oxidation of iodine by the organism. The same flasks used for our preliminary test received an additional dose of ammonium sulphate and after twelve more days of incubation, were removed from the incubator. Urea, and hydrochloric acid were added in equal amounts in all flasks, so as to remove the nitrites by the Piccini reaction (1879), and allowed to stand in the cold for several days to complete the removal of the nitrites. Then, after the addition of barium hydrate solution, they were filtered and the residues washed with cold water several times to remove the traces of nitrites that might be found, due to incomplete reaction. After digesting the residue in weak HCl the mixture was again filtered and the filtrate tested for the presence of iodic acid by the addition of a 0.25 per cent solution of sodium sulphite using starch as indicator. The blue color would in this case only develop in presence of iodates. Tests with four different strains were concordant in showing iodides but no iodates present at the end of such an experiment.

Apparently no appreciable, if any, oxidation of the iodine could be observed in the cultures and it seems likely that in the soil another group of organisms (possibly the nitrate former) is responsible for the phenomenon detected by Müntz. It should be kept in mind however in this connection that Omeliansky (1902) could not prove for this latter group of organisms the presence of a function of oxidation on the sulphurous and phosphorous acids.

IRON AND NITROSOCOCCUS

General

Winogradsky and Omeliansky (1899) found that iron is necessary for the process of nitrification and Kaserer (1911) attempted to find a condition whereby iron could be made more available for the bacterial cells by dispersion as an aluminium-silico-phosphate. In a previous communication from this laboratory (Bonazzi, 1919) it was found, however, that the iron aluminium-silico-phosphate of Kaserer, when added to nitrifying solutions proved of no avail for the hastening of the oxidative action of *Nitrosococcus*.

Remy and Rösing (1911) and later Allen (1919) in a study of the conditions of iron in cultures of *Azotobacter* showed that the presence of colloidal iron was extremely beneficial to the process of nitrogen fixation by this organism. The work of Allen opens up an interesting field in the physiology of bacteria and points to the possible function of iron as an oxygen carrier and as a protective colloid for the precipitated phosphates.

In the present study however iron is considered from another standpoint, i.e., its behavior in the cellular metabolism. In the nitrifying solutions iron is introduced as a ferrous salt. In view of the fact that iron has been suggested as being involved in processes of oxidation (Fenton and collaborators 1894, 1899 and 1900) by acting as a carrier of oxygen for other substances, while itself undergoing oxidation, it was thought that a study of the changes undergone by this element in a nitrifying solution might throw some light on the process of ammonia oxidation.

Experimental

Cultures 344-0, 344-2, 344-3, 344-6, and 344-7 were used. All except 344-0, which was an uninoculated control, were young cultures and in active nitrification when used. Two cubic centimeters of each culture were placed in tubes and acidified with 5 drops of HCl: each tube was then placed in an apparatus from which all the oxygen was removed by means of alkaline pyrogallol. By means of a dropping funnel fitted in the appa-

tus each tube received a few drops of $\text{FeK}_4(\text{CN})_6$ with the results given in table 3. Under these conditions the ferrocyanide yields a blue precipitate of potassium-ferri-ferrocyanide only in presence of ferric salts. At the same time some of the acidified culture received in the laboratory air a few drops of $\text{FeK}_3(\text{CN})_6$ with the results given in the second line of the table.

When old cultures are tested which have oxidized all their ammonia, and have not received a new addition of this compound for some time, no reaction for ferrous iron is obtained.

The blue precipitate obtained with active cultures is sometimes slow to appear and on close observation appears to be constituted of deeply colored blue cellular clumps. The clear unacidified or acidified filtrate from even a very active culture fails to show the presence of either ferric or ferrous iron. Cells washed free of nitrites on Chamberland filters became deeply blue on prolonged contact with $\text{K}_3\text{Fe}(\text{CN})_6$ in acid solution.

TABLE 3

TREATMENT WITH	334-0	334-2	334-3	334-6	334-7
$\text{FeK}_4(\text{CN})_6$	Deep blue precipitate	Very light blue tinge in all inoculated Dark blue color in all inoculated			
$\text{FeK}_3(\text{CN})_6$	No color				

It is thus evident that the iron in the uninoculated solutions undergoes a very profound oxidation whereas in the inoculated it is partly retained in the ferrous form. In addition to this, it should be mentioned that when cultures were started in an Omeliansky solution which had been prepared for so long as to have all its iron transformed to the ferric form, tests of the control uninoculated solutions failed to give a reaction for ferrous iron, whereas the active cultures yielded a decidedly blue cellular mass.

THE PROCESS OF OXIDATION

General

It is not necessary here to review all the theories that have been advanced for the explanation of the phenomena of oxidation since an interesting discussion of this subject may be found in Mellor's book (1909) and Kastle's monograph (1910).

In previous communications from this laboratory it was shown that the presence of a KOH container in a cultural system stops nitrification, and this fact may be correlated with the greater concentration of the free NH_4 ion in the culture solution, resulting in a greater toxicity to the organism. This also stands out from the work of Meyerhoff (1917). To this should be added the action of the absence of all traces of CO_2 in the atmosphere and medium.

Aside from the fact that Omeliansky (1902) failed to isolate an oxidase from the cells of the nitroso organism nothing is known with respect to the intimate mechanism of this phase of assimilation. It is true, Chodat (1906) suggested the existence of a nitroxidase but he really brings forth nothing in the line of experimental proof of his suggestion when he states that since ammonium carbonate is in reality an ammonium carbamate, the nitroxidase is in truth a carbamase, capable of attacking only this type of linking.

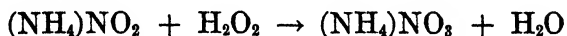
In these processes of oxidation the organism must take an active part by means of an intracellular mechanism since various attempts made in this laboratory failed to demonstrate an extracellular nitroxidase thus corroborating the findings of Omeliansky. An oxidase may be considered as a joint system of peroxide-peroxidase; in the presence of a peroxide, oxygen is liberated by the peroxidase and used in the oxidation of oxidizable substances. The peroxide need not be peroxide of hydrogen, and, as is clearly pointed out by Bach and Chodat, may be an organic peroxide. If a mechanism were found whereby the oxidation of ammonia could be brought about by means of stored energy a step would be taken towards an understanding of the mechanism of the biological oxidation of nitrogen in Nature.

In order to study this question, several attempts have been made in the direction of bringing about an oxidation of ammonia to nitrous acid, in the cold, by chemical means. Weith and Weber (1874) found that ". . . hydrogen peroxide and ammonia reacting upon each other gave rise to nitrous acid. . . ." These authors claimed thereby to have found the origin of nitrous acid in Nature.

Carius (1874) found that ozone and ammonia gave rise to ammonium nitrite as follows



and that



Thus the fact was established that hydrogen peroxide is formed as a transitory product in the reaction, capable of carrying the oxidation one step further. These findings were corroborated by Hoppe-Seyler (1883), Riegler (1897) and McIntosh (1902). However Hoppe-Seyler (l. c.) as well as Griess himself, when recommending his reagent for nitrites (1880), and again Wurster (1886) cautioned against the fact that the presence of hydrogen peroxide interferes with the color development of the naphthylamine and sulphanilic acid reagent for the detection of nitrites.

Fausto Sestini (1904) as well as Russell and Smith (1906) found that a limited oxidation of ammonia could take place in presence of ferric hydrate in an atmosphere saturated with ammonia, and Leoncini and Pieri (1909-1914) found nitric acid to be formed by the action of boiling solutions of manganese dioxid upon urea, cyanamide and dicyandiamide. This last compound gave rise to small quantities of nitric acid even at 30°C.

The conclusions, that these various authors reached, were to the effect that although nitrites and nitrates might appear, the quantities are not important from a practical standpoint. This is true when these actions are considered as taking place independently in the soil, but it is the opinion of the present author that this phase of their importance is secondary. What these phenomena really do mean, is that the active oxygen is liberated from compounds that contain it, and becomes active in the oxidation of ammonia and some organic compounds.

A mechanism whereby peroxides are continually formed and degraded with liberation of the energy stored during the peroxidation, is an oxidase system: a mechanism whereby this peroxide oxygen is used in the oxidation of ammonia becomes extremely

important in the present connection. Experiments were accordingly undertaken to ascertain whether, according to the earlier investigators, nitrites are formed in the presence of peroxides in the cold.

Experimental

To avoid the difficulty of detecting nitrites in presence of hydrogen peroxide the latter compound was precipitated in the form of barium peroxide and the filtered supernatant liquid used for the necessary tests by means of the Illosway-Lunge modification of the Griess reagent.¹

TABLE 4

NUMBER OF FLASK	HOURS OF INCUBATION	CUBIC CENTIMETERS OF SOLUTION USED IN EACH FLASK				RESULT: NITRITE REACTION
		(NH ₄) ₂ SO ₄	MgCO ₃	Na ₂ CO ₃	H ₂ O ₂	
1	0	25	2		5	Negative
2	24	25	2		5	Intense
3	48	25	2		5	Intense
4	0	25		1	5	Negative
5	24	25		1	5	Intense
6	48	25		1	5	Intense

For the experiments on oxidation described in the accompanying paragraphs, 25 cc. portions of a 0.2 per cent solution of (NH₄)₂SO₄, in nitrite-free water, were used together with 1 cc. of a 5 per cent solution of Na₂CO₃ or 2 cc. of a suspension of MgCO₃. Ground-glass-stoppered flasks were used throughout. The peroxide used was a 3 per cent (Parke, Davis) solution and of this 5 cc. were used in each case. The arrangement of the experiments and some of the results obtained are summarized in table 4. The temperature of incubation was 27°C.

Nitrites are thus actually formed under the conditions of experiment. The proof once obtained for this fact, as also of the purity of the hydrogen peroxide used, many other trials were

¹ It was thus found that a 3 per cent solution of H₂O₂ could be made, by means of this procedure, to give a negative test with the ether chromate method for the detection of peroxides. According to Mellor (1912) this test is sensitive to a concentration of one part of H₂O₂ in 80,000 parts of water.

carried out and could here be described. However it is sufficient to state that repeated trials showed that whereas nitrites were formed from the sulphate of ammonia in Omeliansky solution with MgCO_3 , they failed to appear when the carbonate of ammonia was used in absence of a base. Also it was found that a solution of ammonium sulphate in water would not nitrify in absence of a base, whereas the formation of nitrites from a pure solution of NH_4OH and of $(\text{NH}_4)_2\text{CO}_3$ could take place when in absence of magnesium carbonate. The necessity of an alkaline reaction might also explain the failure encountered when $(\text{NH}_4)_2\text{CO}_3$ was used in Omeliansky solution without magnesium carbonate or other base. The peroxide used was in a weakly acid solution.

This formation of nitrites was found to take place also in an atmosphere free of CO_2 although at a rate slower than in ordinary air. Nevertheless even under these conditions of CO_2 removal ammonium carbonate in Omeliansky solution failed to nitrify.

As has been stated, oxidation will take place even in solutions of the pure salts, without addition of other materials, and this is an additional proof that the chemical process is independent of any other constituent of the Omeliansky solution. Experiments with this solution from which the various salts had been omitted one at a time, served to prove this assertion. In every case nitrite was formed, after 24 hours of incubation, except in those cases where MgCO_3 or $(\text{NH}_4)_2\text{SO}_4$ had been omitted.

It would seem reasonable to assume that, if the mechanism of biological oxidation of ammonia is tied up with the action of a peroxide, it should be possible to demonstrate the presence of one of these compounds in the active cultures of the organisms of nitrosofermentation. The fact that the ether-chromate test for peroxides fails to give any results with active cultures, might open the possibility that the peroxide concerned in the biological nitrification is an organic peroxide other than the peroxide of hydrogen. However, the transitory nature of a peroxide in an active culture, or any other oxidizing system, should be here emphasized on theoretical grounds.

Again, an active culture filtered and washed free of nitrites with water, or freed of its nitrites by Piccini's reaction, fails to indicate the presence of an oxidase or even a peroxidase when tested with the guaiac gum solution. Omeliansky relates a similar experience. Another line of attack should then be devised.

Were a peroxide active in the biological oxidation of ammonia, it should be possible to find the mechanism whereby the peroxide oxygen is liberated. Accordingly some experiments were undertaken with the object of establishing whether cultures in active nitrosofermentation are capable of breaking down the H_2O_2 molecule with liberation of oxygen.

For this purpose a small shaking machine was used which imparted a uniform motion to two flasks; one flask containing the living culture the other the control solution. The control solution was in some cases freshly prepared or incubated sterile Omeliansky solution, in other cases a culture in active nitrification which had been pasteurized previous to use. In every case 2 cc. of culture, living or variously treated, were intimately mixed in the flasks with 10 cc. of a 1.5 per cent solution of hydrogen peroxide, and the oxygen developed was collected over water at constant room temperature and measured in graduated burettes at definite intervals.

The experiments carried out, all gave similar results and only a few are given in table 5 as typical examples. The values given in this table are also graphically reproduced in figure 1.

From the results just given it is possible to derive the statement that the bacterial cells in a culture of *Nitrosococcus*, or some of their by-products are capable of bringing about the breakdown of hydrogen peroxide.

By filtering an active culture, washing it with acetone and finally drying it with ether, a white powder is obtained which is principally made up of precipitated magnesium carbonate. When this powder is used, after suspension in water, and compared with another portion of the same material which has been kept at boiling water temperature for ten minutes in a water bath, values are obtained which are given in table 6.

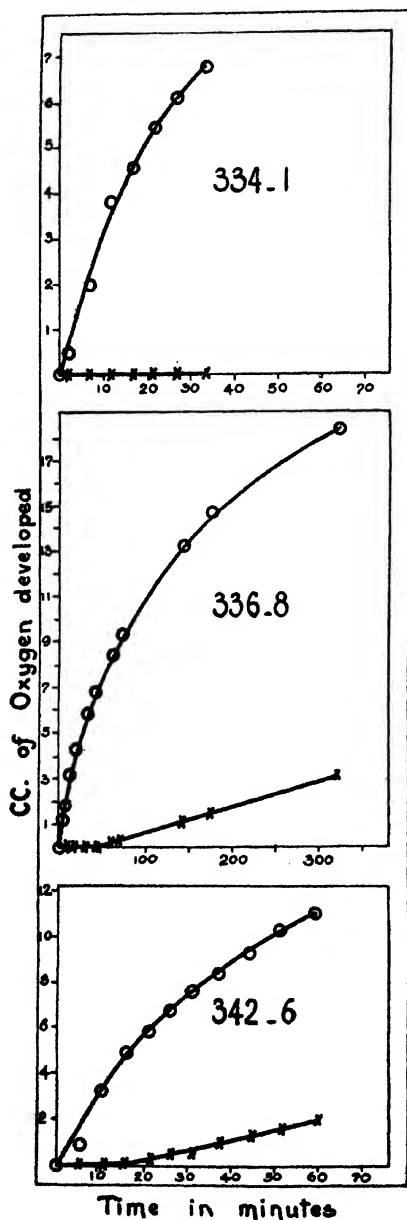


FIG. 1.

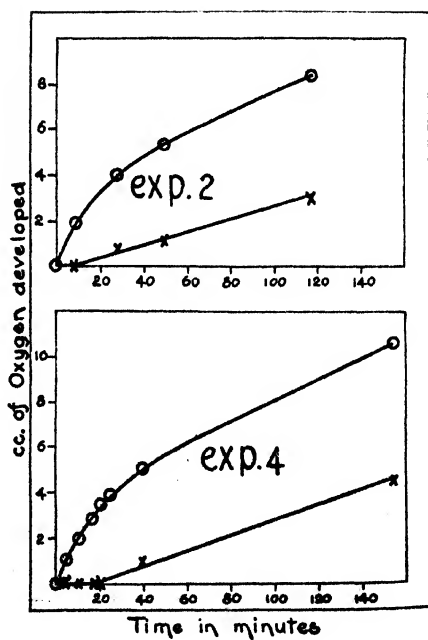


FIG. 2.

The results given in table 6 are graphically shown in figure 2. The acetone-ether preparations used in these experiments had stood for three days in the dry condition. However, when a culture which had nearly finished dissolving its MgCO_3 precipitate, due to the formation of large quantities of nitrous acid,

TABLE 5

CULTURE 334-1			CULTURE 336-8			CULTURE 342-6		
Time in minutes	Oxygen developed in cubic centimeter		Time in minutes	Oxygen developed in cubic centimeter		Time in minutes	Oxygen developed in cubic centimeter	
	Culture	Control		Culture	Control		Culture	Control
0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
2	0.50	0.00	6	1.90	0.00	5	0.95	0.00
7	2.00	0.00	13	3.25	0.00	10	3.25	0.00
12	3.85	0.00	19	4.35	0.00	16	4.95	0.00
17	4.60	0.00	33	5.95	0.00	21	5.85	0.25
22	5.50	0.00	41	6.90	0.00	26	6.75	0.45
27	6.15	0.00	61	8.50	0.30	31	7.65	0.45
34	6.85	0.00	71	9.40	0.40	37	8.45	0.95
			143	13.35	1.25	44	9.30	1.30
			175	14.80	1.60	51	10.17	1.45
			326	18.47	3.25	60	11.16	1.85

TABLE 6

EXPERIMENT 2			EXPERIMENT 4		
Time in minutes	Oxygen developed in cubic centimeter		Time in minutes	Oxygen developed in cubic centimeter	
	Culture material	Boiled control		Culture material	Boiled control
0	0.00	0.00	0	0.00	0.00
8	1.82	0.00	5	1.10	0.00
28	4.00	0.80	11	2.10	0.00
50	5.30	1.17	17	2.85	0.00
118	8.27	2.95	21	3.50	0.17
			25	3.95	0.20
			40	5.10	0.90
			155	10.60	4.55

was centrifuged, washed several times with water, acetone and then ether, poured into a crystallizing dish to dry and subsequently experimented upon, it failed to give any appreciable liberation of oxygen from the peroxide due no doubt to the weak but distinctly noticeable acidity of the reacting system.

Thus the mechanism of peroxide breakdown is truly "catalytic;" it is destroyed by heating and is completely lacking in fresh Omeliansky solution as well as in this same solution to which nitrites have been artificially added. The curves obtained when this solution is used sterile, approach very nearly a linear function and are indicative of the surface action of the system. In the cultures instead, there is a strong activity at first with a subsequent retardation as time goes on, the curve tending to become parallel to the abscissa.

DISCUSSION AND CONCLUSIONS

As has been mentioned earlier in this contribution, peroxide oxygen could not be demonstrated in the cultures even with as delicate a test as the ether-chromate reaction.

Nevertheless a mechanism is undoubtedly developed whereby this compound is broken down with the liberation of free oxygen (a peroxidase reaction) and this leads to the conclusion that this compound or compounds of similar nature play an active rôle in the process of nitrosofermentation. Unfortunately experiments to investigate the action of hydrogen peroxide additions to active cultures have so far given no indications of value, but this is possibly due to the excessive concentration of this compound in the immediate vicinity of the cells.

Iron is found in active cultures partly in the ferrous state and this leads to a special train of reasoning.

When ferrous sulphate is acted upon by an alkali (NaOH) in the absence of air, a white precipitate is formed of ferrous hydroxide which as long as it is kept free of oxygen remains white, but becomes yellow, green and later dark-brown to black in the presence of the smallest traces of oxygen indicating that this compound has changed in structure and has acquired the structure of ferrous oxide-peroxide. This latter compound is far more active both chemically and electrically than the white ferrous hydroxide, and, as was shown by Quartaroli (1917) and by Baudisch (1921) is concerned with many of the changes in the oxygen content of compounds which, alone, are quite stable in ordinary conditions.

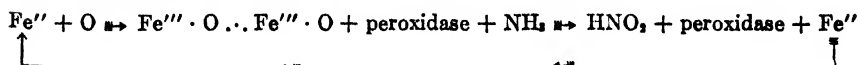
The return of the highly oxidized form of iron to the compound of lesser oxidation, by yielding its oxygen to the bacterial cells would release the energy gained in the first step of oxidation and thus facilitate the inception of the second step, of nitrification proper, which as has been seen, can easily be reproduced by artificial means. By attacking the ferrous-oxide-peroxide the bacterial cells allow nitrification to proceed maintaining the ratio of $\frac{O}{N}$ equal to that found in the present work (± 3).

The iron acting as a carrier of oxygen would be far more active in a shallow layer in continual motion than in a deep layer, thereby hastening nitrification; this has been found to happen in previous work in this laboratory. In this connection it should be mentioned that Ashby (1907) found nitrification taking place very actively in presence of "ferric hydrate" and "iron rust" as the only bases present. The meaning of these results is evident in the light of the knowledge gained in the present investigation. The iron in Ashby's cultures not only acted in virtue of its weak alkalinity, as was argued by this author, but also as an active oxygen carrier. Here the results obtained by Russell and Smith, reported above, are extremely interesting.

The breakdown of the peroxide with the liberation of the "activated" oxygen is accomplished by a mechanism inherent to the cell itself, similar to that exhibited by the cells when in presence of the high concentrations of hydrogen peroxide used in the present study.

Accepting Bach and Chodat's conception of oxidase action, we can conclude that the iron by its mechanism of autooxidation fulfills in the nitrifying cultures the functions of the peroxide, while the cells furnish the mechanism for the liberation of the oxygen thus bound.

A direct proof of the existence of an oxidase by the guaiac test can not be obtained due to the presence in the solution of other compounds (biproduets of the bacterial activity itself) which will themselves oxidise the guaiac gum. Nevertheless it appears probable that the system may be represented by a sequence such as follows.



The ferrous-hydroxide-peroxide plays here the rôle which the peroxide plays in the system of Bach and Chodat.

To this fact may be added the observation made often in the course of these studies that the iron cannot be washed out of the cells even by the action of strong nitric or hydrochloric acids.

The change that nitrogen undergoes in passing from the ammoniacal to the nitrous form is one which involves the valency of the element. From a scientific standpoint therefore, as well as from a practical, the process of biological oxidation of ammonia presents great interest. Lafar (1911) in discussing this question states; “. . . as is apparent from this equation, their power (nitrobacteria) differs from that of the nitrosobacteria, inasmuch as the latter convert the pentavalent nitrogen of ammonia into the trivalent nitrogen of nitrous acid, whilst the nitrobacteria reconvert the element into the pentad condition. . . .”

The fact that the two types of organisms are capable of such widely different functions should be confronted with their similarity of behavior in cultural conditions, a thing that at present finds no explanation.² The question of the autotrophy of the organisms concerned is of importance in this connection and should be correlated with the powers which these two organisms have of utilizing, by means of their cellular complex, the energy changes of the element nitrogen.

Naturally the change in the condition of the nitrogen is subordinate to the mechanism of oxygen activation, since the cultural system itself free of bacteria is inert, from the standpoint of nitrous and nitric acid formation; it should be realized that the

² It is true that according to Chodat the nitrosoferment oxidizes only ammonium carbonate because this compound is really an ammonium carbamate with nitrogen in the trivalent state. The hypothetical nitroxidase of Chodat would only be capable of oxidizing the amino group of this compound, by an action similar to that of the enzymes of the laccase type which oxidize commercial cyanamide; the hydrogen peroxide and carbon here acting as secondary catalysts. However if this were the case, it is difficult to find an explanation for the complete disappearance of the ammonia from a nitrifying system, a fact that indicates that the total molecule of the carbonate has been utilized. Besides the existence of a nitroxidase has not as yet been proven.

simple acceptance of the living bacterial cells as catalysts in the ordinary meaning of the word will not explain the changes that occur during nitrification, unless a study is made of the conditions that allow the bacterial cells to produce and utilize a mechanism for the activation of the inactive atmospheric oxygen. It is the object of this paper to show that the mechanism whereby this phenomenon, preliminary to the process of nitrification proper, and intimately connected with it, takes place is one in which iron is active by virtue of its property of activating the inert oxygen of the air; the mechanism appears to be intracellular. This explains why Omeliansky and the present author failed to isolate a nitroxidase from active cultures of *Nitrosococcus*.

From the practical standpoint the present study finds an application in the new interpretation offered for the process of nitrite and nitrate formation in Nature. The question of autotrophy of the organisms, active in these changes, has for a long time been a question-mark in the study of bacteriology. Various authors have also attempted to show that the autotrophy is only a condition forced by artificial cultural environment. Still no other group of organisms has yet been found which exhibits so marked a plasticity of protoplasmic functions as to become in cultures completely and hereditarily unrecognizable, from a physiological standpoint, from their normal selves.

Undoubtedly, although some authors are trying to make of the group of nitrifying bacteria an aberrant group, highly specialized and derived from the common heterotrophic forms, we ought to consider them instead as very primitive forms, protobacterial so to speak, which retain as yet their autotrophic functions and are active geological agents in the breakdown of the slowly soluble rocks.

The facts often observed relating to the beneficial effects to be derived from the additions of organic matters to impure cultures of the organisms of nitrification, as well as the benefits to be derived from the application of organic fertilizers in agricultural practice, should not be explained by assuming a direct action of these substances upon the organisms themselves, a theory disproved by the work of Winogradsky and Omeliansky

and in fact the only work of the kind ever carried out by means of absolutely pure cultures;³ but by the indirect effect of the contaminating forms on the medium in which nitrification takes place, i.e., the solution of mineral substances which prove necessary to the intimate mechanism of nitrification proper. The findings of Müntz relating to an abundant nitrifying flora in the peaks of alpine mountains are sufficient proof that the above considerations are not far from the truth.⁴

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³ In this connection, a recent note by Winogradsky in *Comp. Rendus.*, **175**: 301, 1922 will be found very appropriate.

⁴ The author wishes to express his thanks to Dr. E. R. Allen, formerly of this laboratory, for his helpful criticisms during the progress of this study.

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FURTHER STUDIES ON THE MORPHOLOGY OF BACTERIA

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Official bacteriology, if I may venture to use the expression, still regards the morphology of bacteria as very simple. The cells of a certain species are supposed to be fairly uniform and to multiply, only by fission. As a matter of fact the very system of classification of bacteria is based on this theory. Many investigations have shown, however, that the theory needs revision. A thorough review of the literature concerning this problem is to be found in the excellent work by F. Löhnis¹ recently issued.

Besides reviewing the literature Löhnis discusses a theory of his own, according to which bacteria develop cyclic changes called life-cycles, including an organized and an amorphous stage. During the latter stage the cell-walls disintegrate and the protoplasm of different cells mixes. From this unstainable "sympiasm" new individual cells develop. If these claims could be proved to be true, it would, I think, be a most interesting discovery, throwing a new light on the problem of variation of bacteria. As is well known, studies of the Twort-d'Herelle phenomenon have shown the great importance of the variation of bacteria. It has been found that not only the lytic agent but the ageing of the cultures induces variation. How does this happen? What rôle do the metabolic products play and how do the hereditary factors work? All these problems are very difficult to approach before we know whether there is any exchange of protoplasm between the cells of the bacteria.

In studying the morphology of *Coryneb. diphtheriae* I have been very anxious to find the structures figured by Löhnis

¹ *Memoirs of the National Academy of Sciences*, vol. xvi, 1921.

especially since Almquist, an author of my own country, describes the same things under the name of "plasmodia." He claims that very small and numerous organized individual cells develop from the "plasmodia." I am able to confirm the statement that the "plasmodia" or "sympiasm" are easily seen ~~for~~ example with *Coryneb. diphtheriae*, in cultures of some age; but I cannot abandon the old view that they are merely slimy agglomerations of cells or, in other words, a "zoogloea." My reasons are that the masses always contain some cells, perhaps empty-looking which, however, are possibly able to grow, and that the production of slime, sometimes in gorgeous masses, in ageing cultures is very common with bacteria. It may be true that the cells growing from the "plasmodia" are sometimes very numerous; but this can be explained in another way, as we shall see later.

Löhnis also describes one mode of interaction between the plasmatic substances of the bacterial cells called "conjunction." This process consists of a "direct union of two or more individual cells" exactly comparable with the copulation of yeasts. The well-known V-forms of *B. subtilis* and *Coryneb. diphtheriae* are the result of such a conjunction, the cells being connected "by bridges or by beaks." With regard to the *Coryneb. diphtheriae* I have followed the development of these bridges and have found that they are not secondary conjunctions, but residues left when the new cell forms from the old one just like the threads connecting the cells of a yeast mycelium.

Nevertheless, though I cannot adduce any evidence in support of Löhnis' discoveries, I am very much inclined to believe in them, since it is improbable that bacteria form an exception to the rule that a species dies at length without sexual rejuvenescence.

Beside the above-mentioned processes Löhnis describes several kinds of asexual multiplication common to all bacteria. One of these is the formation of gonidia. In analogy with the large trichobacteria, as for example *Cladothrix* and *Leptothrix*, bacteria form within the cells small bodies which are able to grow, either while still in the mother cell, or when liberated by the bursting of the cell wall. If the cells containing gonidia have

changed their shape into giant forms they are called gonidangia. The club form of the *Actinomyces* is regarded as such a gonidangium. "Very many of the so-called involution forms of spherical, pearshape, and clubshape are, in fact, gonidangia." I am convinced, however, that many of the pictures explained by Löhnis as growing gonidia are only budding and branching vegetative cells. He says that the great multitude of forms seen with *Bact. coli* and *Bact. typhi* and especially in the pictures of these bacteria published by Hort are due to the development of gonidia before they are liberated from the mother cell-wall. As I have pointed out in numerous earlier papers, these "fantastic" forms are just the same as occur in yeast and must be regarded as normal vegetative cells. A proof that Löhnis exaggerates the importance of the gonidia is to be found on page 144 of his monograph, where he says that "it still remains to be studied whether the eosinophilic granules, which are present in blood-cells, are actually cell-products or bacterial gonidia."

On the other hand, it cannot be denied that fishing from old bacterial cultures, containing no intact cells, or very few, but including masses of easily stainable small granules, often gives an abundance of colonies on the agar plates. It is then very tempting to explain this growth, as many have done, as originating from the granules. But to supply a proof is exceedingly difficult, and I doubt whether it is possible to provide convincing pictures. In diphtheroids and other bacteria I have often seen the organisms in the form of a pure culture of filaments, which in growing old have become more and more granulated, and at last have broken up into granulated masses. Plating from such a culture has, as has been mentioned above, given numerous short bacillary forms. I have supposed that the granules are contracted masses of protoplasm separated by empty spaces in the ageing filament and not definite organized gonidia, since they differ widely in size and shape. The name I have found most adequate is "fragmentation spores" a term used by Bostroem in *Actinomyces*. These formations are, I think, the cause of the striped appearance of *Coryneb. diphtheriae*. They are not to be confused with the polar granules, being real granulas and lying in vacuoles, where they exhibit a dancing movement.

Furthermore Löhnis tells us about some other bodies serving the purpose of multiplication which he calls regenerative bodies. They are of different nature and are produced either by the gonidia or by the symplasm and they are "generally characterized by their being easily and deeply stained by aqueous dyes, by their different appearance as compared with the vegetative cells (globular, oval, or irregular), by their ability to reproduce normal cells immediately or after having propagated as such by fission or by budding, by their distinctly increased resistance against drying, heat, or other detrimental influences." Beside the "regenerative bodies" he describes "arthro-spores" and "microcysts," the latter being formed by "transformation of a whole vegetative cell into one, usually relatively large, resting body" the former "by the segmentation of the vegetative cell and the transformation of these segments into fairly resistant reproductive organs." The thickening of the wall is characteristic of both, but usually the arthro-spores are more cubical and the microcysts more rounded in shape. The microcysts can grow to vegetative cells or change into gonidangia or divide into several equal parts, each able to give rise to a new cell. The author says that "what we now call regenerative bodies is a collection of different reproductive organs, whose proper separation and classification must be left for the time when more data shall be available."

In studying bacteria I have always followed as a guide the theory that bacteria are closely connected with the fungi proper. This theory at once throws light on the pleomorphism of the bacteria and also on many problems of variation. According to this theory, it is natural that the bacteria cells should differ widely in shape and that new variants of more or less constancy, should rise in a culture. In the same way I have tried to explain the structure of the bacterial cell and finally also some peculiar bodies which I have seen in the case of *Coryneb. diphtheriae*, *Bact. coli* and *Bact. typhi*. These bodies evidently belong to one of the above mentioned groups of Löhnis. For my part, however, I should prefer another name to those used by Löhnis since these bodies are most similar I think to the so-called chlamydospores of the fungi. What then is a chlamydospore? This name is

given to a peculiar kind of resting form having thick walls and being a little more resistant than common vegetative cells. Many of the Eumycetes, for example *Favus*, show mycelial filaments with circumscribed swellings or bulges. Such swellings are often transformed into chlamydo-spores by the flowing of the protoplasm from other parts of the filament to the swelling, which afterwards surrounds itself with a thick wall and becomes granulated. Owing to the abundance of protoplasm and the presence of the thick membrane, the body takes stain in a higher degree than the other cells. The remaining parts of the filament soon die and the spore is free. The "resting cells" of yeast show a very great resemblance to these chlamydo-spores. The only difference is that here the whole cell is transformed into a resting form. Chlamydo-spores according to Plaut are demonstrated on the text-figure below. Now it may be mentioned, first of all, that many bacteria show just the same swellings, as is well known to every one familiar with ageing bacterial cultures. Sometimes the swellings are situated in the end of the filament, which then assumes a club-like shape, common to many bacteria, for example, *Mycob. tuberculosis* and *Coryneb. diphtheriae*. In other cases the swelling has a peculiar triangular form. The formations just described are mostly to be seen on pictures of "involution forms;" but many writers have accepted them as organs of multiplication. It may be remembered that Plaut utters a warning against the confusion of these swellings and spores and other higher formations found in fungi. How much more easily does such a confusion take place when we are dealing with bacteria which are of so small a size.²



FIG. 1. CHLAMYDOS-
PORES ACCORDING TO
PLAUT

Sometimes, however, you will see the whole development of chlamydo-spores from these swellings even in the case of bacteria. *Bact. typhi* shows these forms, especially when grown at low temperature and on drying agar as Almquist has pointed out, and

² In Kolle-Wasserman, Handl. der path. Mikroorganismen, Bd. v, Jena, 1913.

Coryneb. diphtheriae shows the same forms when left for a week or more on Löffler serum. Figures 8, 12 and 16 demonstrate such bodies from a culture of *Bact. typhi*. As to *Coryneb. diphtheriae* I refer to an earlier article. Neither of these bacteria, however, is very suitable for studies of these formations, since they appear rather irregularly and only in ageing cultures. I have found excellent material in a *Corynebacterium* originally isolated by Rosenow from a case of Hodgkins disease and kindly provided me by R. R. Mellon. The corpuscles appear in the cultures of this bacterium in great abundance even after twenty-four hours' growth on plain agar as is to be seen on figures 1, 7 and 13. A scrutiny of the living microorganism under high magnification showed that the filaments produced swellings which, just like those of *Favus*, were situated everywhere, even in the ends, which then looked like the "clubs" of *Coryneb. diphtheriae*. Thickening of the cell-wall and gathering of the protoplasm inside was clearly seen in many of the swellings. On the other hand, such bodies were also seen free, with only faint traces of the filament attached to them. These traces and bodies on the ends of branched filaments prove that the development is that hinted at above and not the reverse, as many have imagined. As a matter of fact the pictures obtained from the development of the chlamydo-spores in a hypha is sometimes strikingly similar to those of the outgrowth of a spore to a filament, especially when dealing, not with long filaments, but with rods or bacillary forms and when the body is placed at the end. But the observation of bodies in both the ends of the rod immediately solves the question. As far as I can see, Meirowsky³ has made such a mistake in interpreting his pictures of *Mycob. tuberculosis*.

But those bodies, are they not gonidangia, asci, or whatever you like to call them? With the very same microorganism Mellon⁴ has found bodies containing small motile corpuscles getting free by the bursting of the wall and giving rise to new individuals. I have not been able to cultivate these bodies; and

³ Studien über Fortpflanzung von Bakterien, Spirillen und Spirochäten, Berlin, 1914.

⁴ Journ. Med. Research, 1920, 62, 61.

they are not the same as those described above. The chlamydospores, as I like to call them, never contain any formations indicating a multiplication, and they grow by budding just as yeast does. The evidence of budding was easy to obtain, since the bodies put in new broth and removed from this in a high dilution of thionin showed beautiful "budding discs" just like those of yeast and other microorganisms described by me in an earlier paper.

Finally I want to emphasize the fact that I do not deny the existence of other more complicated forms in bacteria, but merely report what I have seen and have not seen. In vain I have tried to find gonidangia or asci. Even with *V. cholerae*, where large round, thick-walled granulated cells very suspiciously similar to the organs of multiplication, are common in ageing cultures, I have never found anything of that kind. On figures 2, 4 and 5 some of these bodies of *V. cholerae* are demonstrated. It is, as will be seen, big enough to be easily studied.

SUMMARY

The author discusses several theories concerning peculiar forms in bacteria supposed to be organs of sexual and asexual multiplication. He also describes some spherical, ovoid or club-shaped bodies found by himself with *Bact. typhi*, *V. cholerae*, *Coryneb. diphtheriae* and other species, which he regards as chlamydospores.

EXPLANATION OF THE PLATE

Figures 3, 6, 9, 14 and 15 are taken from wet preparations obtained by transferring the microorganisms from the culture to a drop of the staining fluid, which was then covered with a glass. In such a thin layer of fluid the microorganisms lie motionless at least for a time long enough to allow photographing. The other pictures are taken from preparations fixed by drying the slides in the usual way. The staining fluid was in all preparations a highly diluted solution of thionin in water.

Figures 1, 7 and 13 (*Corynebacterium lymphogranulomatis* Rosenow). Slanted agar culture twenty-four hours old. Many chlamydospores in different stages of development.

Figures 3, 9 and 15. The same bacterium with a chlamydospore developing near or in the end of a hypha. In the two latter the microorganisms have become more or less club-like.

Figures 6 and 14. Development of the spore in the middle of the hypha. The swelling of the thread, the thickening of the wall, and the gathering of the protoplasm to the spore are clearly seen.

Figures 10 and 11. Free spores, the former still with remnants of the thread adherent to the wall, as is mostly the case.

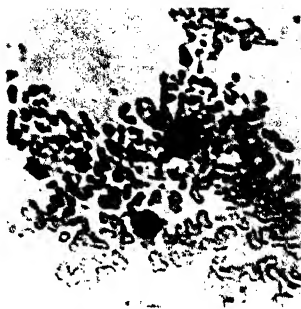
Figures 2, 4 and 5. Round bodies in a culture of *V. cholerae* on slanted agar three weeks old. In figure 2 the size of the bodies can be compared with that of the usual form of the microorganism. In figure 5 the body shows a definite granulation. The bodies are supposed to be resting cells.

Figures 8, 12 and 16. Pictures from a slanted agar culture of *Bact. typhi* three weeks old. In the two latter a chlamydospore is seen to develop at the end of a rod, giving to this a club-like shape. The next stage, with the rod shrinking away and the spore more or less liberated, can be observed on figure 8.

Plate I



1



2



3



4



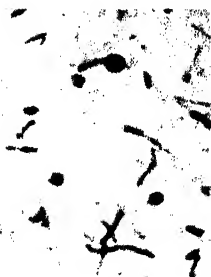
5



6



7



8



9



10



11



12



13



14



15



16

(Bergstrand: The Morphology of Bacteria.)

CLOSTRIDIUM PUTRIFICUM

II. MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL STUDY

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Interest in this peculiar but extremely important organism has increased within the past few years. Since Sturges and Rettger (1919) brought out again the outstanding features of this anaerobe, among them that of delayed development in pure culture, investigators have expressed widely different views. An example of the uncertainty concerning the entity of the organism in question is given by the British Medical Research Committee. In their first report on the classification and study of the anaerobes found in war wounds (Special Report Series, No. 12, 1917) certain cultural reactions attributed to it are given rather fully and there is every reason to assume that the committee at that time believed in the existence of a definite species which they were calling *Bacillus putrificus*. Later (1919) in their Special Report Series, No. 39., they do not make reference to it as a distinct species, but infer that "many of the cultures of *B. putrificus*, so-called, are really mixtures of *B. cochlearius* or *B. tertius* with *B. sporogenes*." They make no effort to list the cultural characters of the organism, and simply offer a short review of the work of previous investigators, which does not identify it in any way as a distinct species. The Lister Institute (London) does not list cultures of *C. putrificum* in its National Collection of Type Cultures (1922) and the writers were unable to procure from them any cultures of this organism for comparative studies.

Hall has, in a way, shown equal uncertainty in regard to the identity of *C. putrificum*. In the differential key to the sporulat-

ing obligate anaerobes given in Jordan's General Bacteriology, this key being taken from some of Hall's unpublished data, Jordan puts *C. putrificum* in a group separate from *C. cochlearius*. The basis of differentiation is a very fundamental one, that of liquefaction of coagulated albumen and blackening of brain. Following close upon the publication of this classification scheme, however, Hall (1922) specifically states that his studies show that *C. putrificum* and *C. cochlearius* are identical. Hall, in the latter work at least, was evidently working with pure *C. putrificum*, but the descriptions are incomplete and in some respects erroneous. This is especially true regarding growth in milk, in which he reports no visible action. The description of *C. cochlearius* given by the British investigators is very much at variance with that suggested by Hall, the Research Committee claiming it to be identical with *C. putrificum*. The writers made a preliminary examination of the type strain of *C. cochlearius* received from the Lister Institute and found it to correspond to the description given in the British Report No. 39, and to be very unlike *C. putrificum*.

It must be admitted that many of the investigators working with this anaerobe have made use of impure cultures, and often have not had the real *C. putrificum* in the cultures described. The writers make no original claims as to the use of pure culture: they are interested here merely in defending the specific entity of this anaerobe, and wish to offer complete proof to substantiate their point.

The bacillus of Bienstock is an anaerobe which has constant characters and which has been described a number of times in undoubted pure culture, but each time quite incompletely. Since Bienstock's original cultures were impure, his first descriptions (Bienstock, 1894, 1899, and 1901) are worthless, except in one particular: it is certain, from his descriptions, that he had reference to an anaerobe having round, terminal spores, and that this mixture of organisms caused putrefactive decomposition of meat. Tissier and Martelly (1902) were evidently working with pure cultures, for they emphasize the inability of *C. putrificum* to attack sugars, even glucose, except very slightly

and with the production of such small amounts of acid that the medium never becomes distinctly acid. This characteristic was confirmed by Rodella (1905) and also by Bienstock himself a year later (Bienstock, 1906). Distaso (1911) specifically brings out the characters of *C. putrificum* as given by Bienstock in 1906 and designates his various strains as belonging to the tetanus group, the round, terminal-spore group. Distaso, however, described two species, one of which is the true *C. putrificum*, while the other resembles in some respects both *C. putrificum* and "*B. paraputrificum*" (Bienstock). His *B. putrificus-filamentosus* has the specific properties claimed by Bienstock, and approaches in almost every detail the strains isolated by Sturges. His description, however, is incomplete, and is not sufficient to establish the specificity of the organism.

On the other hand, a very large number of workers have described cultures which are undoubtedly entirely different from the type strain of Bienstock. Among these may be mentioned Metchnikoff (1908), Aschoff (1917), Medical Research Committee (1917 and 1919), Kendall, Day and Walker (1922) and Kahn (1922). The misunderstanding concerning this much-buffed organism has been due in a large measure to the meagre descriptions given in the literature. Hall's work (Hall, 1922) not only is incomplete, but there are some errors which would certainly complicate identification if allowed to stand in the present form. The writers (1921) in a preliminary paper, attempted to point out the true characters of *C. putrificum* and later (1922) published such of their data as would establish the specific entity of the organism. The need for a complete tabulation of the morphological and cultural characters is yet felt. In the present work these are given detailed consideration. Biochemical studies of the action of the organism on peptone and glucose are included to support the qualitative cultural observations. Strains of *C. putrificum* isolated by Sturges were used in this study.

For the sake of clearness, the exact technique employed will be given. This feature is often neglected and the reader has too little information upon which to make comparative studies.

Only ordinary laboratory media were used and the simplest technique employed. The composition of the media and the methods used will be given under each separate heading, or references cited which furnish the particular information.

MORPHOLOGY

The morphology of *C. putrificum* may be said to be characteristic. At least it differs very considerably from the other common anaerobes, with the possible exception of Bienstock's "*B. paraputrificus*." It is a long, slender rod, quite often curved, and in old cultures frequently occurs in long filaments. It is much more slender than the other anaerobes, being generally from 0.5 to 0.7 μ broad and from 7.0 to 8.0 μ long. There are often shorter forms, but these are not the common type. The spore is round and strictly terminal, and very large in proportion to the rod (See Reddish and Rettger, 1922, plate I, fig. 1). In egg-meat medium (see Rettger, 1906), in which the organism grows and preserves its viability particularly well, and in which our observations as to morphology were made most frequently, spores are not to be seen until about the tenth day or after. It may require two or three weeks for the typical spores to appear in appreciable numbers in this medium. The appearance of spores accompanies the beginning of apparent digestion of the meat. The spores remain in the rods for several days after they are first observed. At this stage they are present in large numbers. Hall (1922) apparently failed to note numerous spores in any of his cultures. This may have been due to his using a less favorable medium or not allowing sufficient time to elapse before the examinations were made. No spores have been observed by us on glucose agar and in glucose broth, although no persistent efforts were made to demonstrate their presence in these media.

C. putrificum is weakly Gram-positive, even in young cultures, and may be said to be on the border line between Gram-positive and Gram-negative. It is almost uniformly Gram-negative in old cultures. It stains well with the ordinary aniline dyes. In studying the staining property, various kinds of media were used,

but cultures in egg-meat medium have proved in our hands to be most reliable for this purpose.

Relatively young egg-meat cultures are actively motile, and motility is not completely lost during sporulation.

COLONY FORM

Surface colonies on glucose agar (1 per cent glucose, 1.6 per cent agar, 1 per cent Difco peptone, and 0.5 per cent Liebig's beef extract)¹ even after prolonged incubation are small, delicate and almost transparent. The edge is irregularly, but not deeply dented; there are no outgrowths and no apparant fringe. The structure appears to be somewhat granular, but not coarse. (See Reddish and Rettger, 1922, fig. 2). The aid of a hand lens is necessary in order to see the colonies distinctly, since they are by far the most delicate formed by any of the anaerobes studied by the writers. At least three days are required for the colonies to show any appreciable development.

Depth colonies in glucose agar tubes (1 per cent glucose in 1 per cent nutrient agar) do not appear until the third or fourth day, and then they are mostly in the extreme lower portion of the tube. For the study of deep colonies, inoculation was made at the surface of the medium with a pipet, using a plain broth culture of the organism, the mixing being accomplished by rolling the tube between the hands; the tubes were incubated aerobically. Apparently the conditions in deep agar tubes favor rapid development better than surface cultivation. The development of colonies in the deeper portion of the medium only signifies the strict anaerobic conditions required. The nucleus of the depth colony is dense, and there are fine, but not long, hair-like radiations from the central portion. Because of the indistinctness of the depth colonies, sharp observations could not be made.

¹ Plates poured with a thick layer of this medium were allowed to dry, inverted, at 37°C. over night and then streaked with a bent glass rod from a saline suspension of an egg-meat culture of the organism. Incubation was at 37°C. for three and for ten days under strictly anaerobic conditions. The time allowed for incubation before removal for description was ten days.

CULTURAL CHARACTERS

Egg-meat. Freshly sterilized egg-meat medium was cooled quickly after sterilization, inoculated immediately from ten to fourteen day egg-meat cultures of the organism, and incubated aerobically at 37°C. There was little change, aside from a slight turbidity, until a week to ten days after inoculation, depending upon the amount of inoculum used. By this time there is usually slight softening of the solid matter and the meat and egg particles become finer. This disintegration is at first most apparent in the upper portion of the medium, but latter progresses into the lower layers. Reddening of the meat has by this time become very pronounced. By the end of the second week there is some decrease in the bulk of the solid matter and digestion is very apparent; this pronounced change is accompanied by the evolution of the foul odors that are characteristic of real putrefaction. Because of the evaporation of the liquid resulting from the long incubation, there is never a very deep layer of liquid over the undigested residue, such as is noted in egg-meat cultures of *C. sporogenes*. The meat is darkened only slightly, the deep red color being dominant throughout the digestion. Only when the meat has become entirely decomposed is there definite browning; the residue in old dried cultures is, however, almost black, but this change does not take place until after several months. There is no apparent gas production at any time. The digestion of the egg-meat is never rapid, and the evaporation of the supernatant liquid almost keeps pace with it.

Milk. Deep tubes of freshly sterilized skim milk were cooled quickly in water and then inoculated with one loopful of an egg-meat culture of the organism,² after which sterile melted paraffin was added to form a seal about half an inch in thickness. The tubes were incubated at 34°C, and not 37°C. because the paraffin softened at the latter temperature. Under these conditions there is slow precipitation of the casein and later almost complete

² A loopful of a ten to fourteen day meat culture is as a rule large, for the solid particles cling to the loop in the form of lumps of partly digested meat, in which much enzyme is carried.

digestion. The time required for this action varies with the inoculum, but more definitely with the age of the culture from which inoculation was made. This is illustrated by the following experiment. Four freshly sterilized deep tubes of milk were inoculated with a large loopful of six-weeks old egg-meat cultures of four strains of *C. putrificum*. Four other deep tubes of freshly sterilized milk were inoculated with a large loop of twenty-four-hour egg-meat cultures of these same strains. All eight tubes were sealed with sterile melted paraffin and incubated at 34°C. The four tubes which were inoculated with the old (digested) cultures showed slight digestion at the end of twenty-four hours, and practically complete digestion of the casein within four days. On the other hand, the tubes inoculated with the young cultures did not show any change until the twelfth to fifteenth day, when there was some digestion of the slightly precipitated casein. One of the tubes did not show any signs of growth for four weeks, after which time there was slow digestion of the casein. In all of the last four tubes the digestion took place slowly. The rapidity of the action in the first set was probably due to the preformed enzymes carried over.³

Broth. Ordinary plain nutrient broth made with beef extract was inoculated with a large loop of a three to four weeks old egg-meat culture and covered with sterile melted paraffin. Tubes without paraffin were placed for comparison in an anaerobic jar and both sets were incubated at 34°C. The growth after two days was slight to fair. In glucose broth the growth is little if any better. The turbidity is never sufficiently great to cause at most more than a very slight sediment.

Gelatin. When gelatin is inoculated with a three to four weeks old culture, rapid liquefaction takes place (one to five days), but when young cultures are used as inoculum the liquefaction is correspondingly slow, and may require from two to three weeks. Liquefaction progresses at about the same rate in plain and glucose gelatin.⁴

³ This may account for the varying results claimed with this medium. Some investigators report rapid digestion of casein, while others claim that there is no apparent action at all in milk.

⁴ Deep, narrow tubes incubated aerobically.

Loeffler's serum. Ordinary Loeffler's blood serum used for growing *Coryneb. diphtheriae* was employed in place of the inspissated horse serum recommended by certain English investigators. Growth was observed after three days' incubation at 37°C. in an anaerobic jar, following heavy inoculation with old cultures. Digestion did not take place completely until after five days.

BIOCHEMICAL REACTIONS

Fermentation tests. The following substances were used in testing for acid and gas:

Pentoses: xylose and arabinose.

Hexoses: glucose, levulose, galactose and mannose.

Disaccharides: lactose, sucrose, maltose and trehalose.

Trisaccharides: raffinose and melezitose.

Polysaccharides: soluble starch, dextrin, glycogen and inulin.

Methyl pentose: rhamnose.

Glucosides: salicin, amygdalin, esculin and inosite.

Alcohols: mannitol, glycerol, erythritol, dulcitol and sorbitol.

These were used in plain nutrient 1 per cent agar, adjusted to pH 7.0 before sterilization. The test substances were added to make a 0.5 per cent solution and the medium filled into tubes which had been previously sterilized, thus allowing for a shorter sterilization period. The tubes were sterilized at 15 pounds for ten minutes, cooled quickly to 45°C. and inoculated as soon as possible. Inoculations were made with a 1 to 1 suspension of a twenty-four hour plain broth culture of the organism, 0.5 cc. being added at the surface of the medium. The tubes were then rolled vigorously between the hands to insure thorough distribution of the organisms, and incubated aerobically at 37°C. A control plain agar tube was also included. None of the test substances were apparently fermented. At least there was no visible gas production and tests for acid by the brom-thymol-blue method were negative. In testing for acid the indicator was added to the melted culture after a week's incubation. Colonies were easily visible by the third or fourth day in all of

these media. As is shown below, glucose is probably feebly attacked, but not enough to yield acid in sufficient amount to show in the brom-thymol-blue test as used above.

Glucose-consuming power. One per cent glucose broth was used for determining quantitatively the amount of glucose consumed by *C. putrificum*. The broth was filled into large tubes, 20 cc. per tube, sterilized and cooled quickly before inoculation with 0.5 cc. of a twenty-four hour plain broth culture.⁵ Six tubes were so inoculated and quickly placed in six separate anaerobic jars. In this way it was possible to open one tube at a time without disturbing the anaerobic conditions under which the others were kept. Quantitative determinations of glucose were made by the method of Benedict (see Cole's Practical Physiological Chemistry, 1919, p. 127). Readings were made at the end of 12 hours, 36 hours, 60 hours, 7 days and 14 days. The hydrogen ion concentration of the cultures was also determined at the same time.

Glucose is attacked but slightly, and not enough to be considered as indicative of definite saccharolytic property on the part of the organism. A loss of only 0.1 per cent glucose is recorded (See Reddish and Rettger, 1922, chart 1). Allowing for experimental error, the amount consumed may be even less. At least, the ability of the organism to consume this sugar is not an outstanding or important feature of its metabolism. The hydrogen ion changes did not keep pace with the glucose reduction, due to the simultaneous production of alkali. The pH dropped from 7.3 to 6.6, but the degree of acidity is not such as would be readily measurable in melted agar cultures by any of the tests now employed, as noted above. The amount of chemically determined glucose was reduced from 0.727 to 0.625 per cent in fourteen days at 37°C. *C. sporogenes*, on the other hand, reduced the glucose from 0.727 to 0.385 per cent in fourteen days. *C. tetani* consumed more dextrose than did *C. putrificum*, reducing it from 0.727 to 0.541 per cent in fourteen days. Therefore, relatively speaking, at least, *C. putrificum*

⁵ The use of a twenty-four hour seed culture was made possible by making a heavy inoculum in the original plain broth.

is in a class by itself so far as its action on glucose is concerned. For all practical purposes, it may be considered non-saccharolytic.

Peptolytic property. For determining the degree of peptolytic action on commercial peptone, ordinary plain broth, in 35 cc. amounts in large tubes, was inoculated with 1 cc. of a twenty-four hour plain broth culture. The inoculation was made immediately after sterilization and cooling, and the tubes were placed in separate jars and put under anaerobic conditions, the number of tubes and jars being sufficient to allow for determinations at the end of 12 hours, 36 hours, 60 hours, 7 days and 14 days, without disturbing the unused tubes. The following tests for following the destruction of the peptone were used in the present studies: (1) quantitative biuret, (2) Sørensen, (3) ammonia, and (4) amino-acids. The biuret test is essentially that employed by Vernon (1903). A modified Sørensen test,⁶ as used by Kendall and Walker (1915) and others was used for determining approximately the ammonia and amino-acids present. The use of the ammonia test has been widely employed both in this country and abroad. The test as used in this work makes use of the iodine-thiosulphate titration (see Sutton's Volumetric Analysis, 8th edition, 1900). Van Slyke's modification of Folin's test was employed for liberating the ammonia in the broth cultures, the gas being collected in acid solution of known strength. The iodine-thiosulphate method of titrating was used because of its greater accuracy. The amino-acids were determined by the micro-method of Van Slyke (1913-1914, 1915).

The albumoses peptone, and higher polypeptids are attacked very little before the third day, but by the seventh day there is considerable change. There is, by this time, a great decrease in the biuret-giving substances, with a consequent increase in the ammonia and Sørensen figures. After one week's incubation the ammonia and Sørensen figures increase only slightly over the seven day reading and there is practically no further destruc-

⁶ In the Sørensen test 5 cc. of neutral formaldehyde were employed and the titration with $\frac{N}{20}$ NaOH made immediately after the formaldehyde was stirred into the solution.

tion of the biuret-giving substances. The delayed action on peptone is not surprising, because of the general tendencies of the organism toward poor development in pure culture in the early stages of growth. Since peptone is more easily attacked than the more complex protein of meat, the changes noted as taking place in seven days are comparable to those taking place in meat after a longer period. Another index of the destructive activity of the organism and its enzymes on egg-meat and proteins in general is the almost complete conversion of the amino-nitrogen into ammonia-nitrogen in the experiments. (see Chart 2, in the previous paper, 1922).

Pathogenicity. A 1 to 10 suspension of a two weeks old egg-meat culture when injected intraperitoneally causes no changes whatever in the condition of the animals tested (white mice and guinea pigs).

Although the media and methods used in this study have been very simple, sufficient data are given to point out clearly the essential features of this peculiar organism. The characteristics as brought out here coincide with certain outstanding features ascribed to it by Bienstock. It is hoped that in the present work, the characteristics exhibited and described may be of material use in placing the organism in its proper group in the classification of anaerobes, and also offer a more or less complete method of identification with the kinds of media most used in this country. A definite effort has been made to employ only such media as can be procured and made easily.

According to the work here presented, *C. putrificum* is not only a definite and distinct species, but should be placed in a group of its own among the known anaerobes. It is proteolytic, peptolytic and at most only very slightly saccharolytic.

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THE TITRIMETRIC ADJUSTMENT OF THE HYDROGEN ION CONCENTRATION OF BACTERIOLOGIC CULTURE MEDIA

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Adjustment of the reaction of bacteriologic culture media to a definite hydrogen ion concentration has seemed such a difficult procedure that some laboratories have not even yet given up the inaccurate and obsolete method of adjustment by titration which was standard a few years ago. Yet that method was fundamentally sound if only an indicator whose range included the desired end point had been used.

Some bacteriologists have tried, and it is a notable tendency among students to attempt, to translate the nomenclature of the older method into that of the newer one, but this is possible only with unbuffered acids and alkalis or in solutions of known and unvarying buffer content, and is manifestly impossible for the organic mixtures in use for cultivating bacteria. Even "standard" formulas are likely to involve important variations in the chemical composition of their ingredients that affect the hydrogen ion concentration of media made with different lots.

Bacteriologists seem generally also to have assumed, incorrectly, that adjustment of a culture medium to a definite pH value necessarily involves the predetermination of the hydrogen ion concentration of the medium, and as a result adjustment by the colorimetric method has generally been based upon what is

actually titration, but titration in tubes¹ with a comparator block rather than in the more convenient evaporating dish or beaker.

It is the purpose of this note to emphasize (a) that a culture medium may be adjusted to a definite hydrogen ion concentration without actually determining its initial pH value, (b) that the amount of acid or alkali required to adjust may be titrated by a procedure almost identical with the old standard method, using beakers or evaporating dishes, and (c) that meticulously standardized (i.e., normal) acid and alkali are not required.

Clark has repeatedly, and particularly in his recent book,² mentioned that adjustment of the reaction of culture mediums is essentially a matter of titration, but McCrudden³ has given what seems to me the most cogent statement of procedure yet published, as follows:

To adjust media to any desired hydrogen ion concentration, $\frac{N}{10}$ alkali is added drop by drop to 5 cc. of the somewhat diluted media containing indicator until, as shown by comparison with the standards, the desired hydrogen ion concentration is reached. From the amount of alkali required for 5 cc. the amount needed for the whole batch of media can then be calculated. Sterilization of the media shifts the pH about 0.2 towards the acid side. Allowance should be made for this.

But McCrudden advocates the comparator, which, after all, is considerably more complicated in its use than a pair of evaporating dishes or beakers.

¹ Hurwitz, Meyer and Ostenberg, On a colorimetric method of adjusting bacteriological culture media to any optimum hydrogen ion concentration, *Proc. Soc. Exp. Biol. and Med.*, 1915, **13**, 24.

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² Clark, *The Determination of Hydrogen Ions*, Williams and Wilkins Co., Baltimore, Maryland, 1920.

³ McCrudden, The determination of hydrogen ion concentration, *Pub. Health Reports*, 1922, **37**, 334.

We in this laboratory have for some time been using and teaching a method of titrimetric adjustment of hydrogen ion concentration of culture media that is really simpler than the method of adjustment to a particular titratable normality.

For general purposes we assume that a pH value of 7.0 approximates the optimum reaction. The same principles of adjustment apply, of course, to any other pH value, using appropriate indicators.

To adjust a medium of unknown reaction to pH 7.0, determine first whether it reacts as an acid or as a base to brom-thymol-blue by adding 0.3 cc. of a 0.04 per cent solution of this dye in 50 per cent alcohol to 10 cc. in a white evaporating dish or a beaker against a white background. A yellow color indicates an acid medium, a blue color indicates an alkaline medium, and a green color indicates a neutral medium. Highly colored (brown or yellow) alkaline media may also appear green and should be diluted so that the normal color of the medium does not interfere with that of the dye.

If the medium is acid, ascertain the number of cubic centimeters of weak alkali required to match the color of the medium with that of an equal amount of a standard buffer solution carefully adjusted to pH 7.0 and containing the same amount of indicator in a similar vessel.

If considerable dilution of the medium and indicator occurs either in obliterating the original color of the medium or in titration, it should be compensated in the standard by an equivalent addition of distilled water.

It is not necessary that the alkali used in titration or in adjustment be standardized in terms of normality; but the relative strength of titrating fluid and adjusting fluid must be known. It is generally convenient to use approximately $N/1$ NaOH for adjustment and an exact dilution of 1:100 for titration; the same number of cubic centimeters of the titrating solution required to neutralize 10 cc. of medium should be used of adjusting solution to neutralize each liter. A check test should be made after the adjustment is complete, and again after sterilization. The final hydrogen ion concentration of the finished medium should be recorded.

No general rules can be laid down as yet regarding the correct allowance to make for changes in reaction during sterilization but any careful technician by repeated observation of the changes that occur in the different types of medium ought soon to establish a set of working rules to guide procedure in his own laboratory.

Certain observations upon the titrimetric method of adjusting hydrogen ion concentration are of interest.

The standard buffer solution must, of course, be made up in the first instance with extreme care. Those that I worked with were prepared by Foster and Randall¹ and were carefully checked by the electrometric method. Such buffer solutions are apparently little changed by ageing in good glass bottles; even the paraffine lining of the bottles which often parts from the glass during warm weather and allows free contact between solution and bottle is probably superfluous. But I wish particularly to emphasize that subsequent lots of a given buffer solution that is almost exhausted may be made simply and easily by the titrimetric method, using the residue of the lot to be replaced for the color standard, without the tedious recrystallization of salts and redistillation of water required for the initial standard.

Furthermore, in the use of the buffer solution as a color standard, our ordinary distilled water, which reacts distinctly acid to brom-thymol-blue, had no apparent effect upon the green color of our pH 7.0 buffer even on dilution of 1 part of buffer to 20 parts of water; at 1:40 however there was a slight difference in the direction of acidity. It is apparent therefore that considerable economy of standard buffers may be effected by such dilution.

The question of the proper temperature for titration received answer in experiments involving undiluted and diluted (up to 1:20) buffer (pH 7.0) similarly tinted with brom-thymol-blue. Boiling indicates a slightly higher alkalinity, presumably through driving out CO_2 . But since the green color returns similarly on cooling either to the temperature of incubation ($37^\circ\text{C}.$) or to room temperature, titration without boiling is advocated as a routine practice.

The amount of dye to use may be varied within rather wide limits according to the desire of the titrator for while the *quantity* or intensity of color depends directly upon the amount used, the

quality of the indicator color of brom-thymol-blue is essentially the same when used in quantities of from 0.1 to 1 cc. of 0.04 per cent alcoholic solution in 10 cc. of our standard buffer (pH 7.0) diluted or undiluted. It is of course essential to have the same concentration of dye in the medium or solution being titrated and in the standard.

I have paid particular attention to the feasibility of diluting the medium for the purpose of obviating color interference. If results obtained with an almost colorless medium (meat infusion broth with 2 per cent peptone and 0.5 per cent NaCl) can be considered as applicable to one with more color, it seems, firstly, that the hydrogen ion concentration reading is unaffected by dilution of 1 part medium with 10 parts distilled water, notwithstanding that the water alone showed a markedly yellow color (see table 1) at room temperature and a strikingly blue color on boiling. This statement certainly holds also for mediums ranging in pH value from 6 to 8.

TABLE 1

*The effect of dilution of medium upon the hydrogen ion concentration reading**

	COLOR COLD	COLOR ON BOILING
10 cc. medium undiluted.....	Pale green	Slightly yellow
3 cc. medium plus 7 cc. distilled water	Pale green	Slightly yellow
1 cc. medium plus 9 cc. distilled water	Pale green	Slightly yellow
10 cc. distilled water undiluted.....	Yellow	Blue

* Each mixture contained also 0.3 cc. 0.04 per cent alcoholic solution of brom-thymol-blue.

Clark and Lubs¹ have shown both mathematically and experimentally that considerable dilution of a buffered medium is permissible before the hydrogen ion concentration is visibly altered.

On the other hand, several experiments have shown that the amount of alkali calculated as required to adjust media that were too acid, to a pH value of 7.0 is greater when such calculations are based upon the titration of samples diluted too far. In other words, a comparison of separate lots of a medium ad-

justed upon the basis of titration with varying dilutions of the medium always showed that those adjusted by titration of too highly diluted samples were made too alkaline. The differing quantities of alkali so calculated in one such experiment are shown in table 2.

In these experiments it was always observed that as the buffer in the medium was decreased by dilution the effect of each drop of titrating fluid became more marked, i.e., the indicator became

TABLE 2
*Effect of dilution of media on amount of alkali required to adjust to pH 7.0**

	OBSERVED N/100 NaOH REQUIRED TO MATCH STAND- AT pH 7.0	FACTOR	CALCULATED N/1 NaOH REQUIRED TO ADJUST 1 LITER	COLOR OF ADJUSTED MEDIUM 100 cc.
	cc.		cc.	
10 cc. medium undiluted.....	9.0	1	9.0	Green
5 cc. medium plus 5 cc. distilled water.....	4.4	2	8.8	Green
3.3 cc. medium plus 6.6 cc. dis- tilled water.....	3.2	3	9.6	Bluish green
2.5 cc. medium plus 7.5 cc. dis- tilled water.....	3.0	4	12.0	Blue
2 cc. medium plus 8.0 cc. dis- tilled water.....	2.3	5	11.5	Blue
1 cc. medium plus 9 cc. distilled water.....	1.2	10	12.0	Blue

* Each mixture contained also 0.3 cc. 0.04 per cent alcoholic solution of brom-thymol-blue.

more sensitive to equal increments of titrating fluid. Since exactly similar results were obtained with boiled and unboiled distilled diluting water, and the acidity of the water was insufficient of itself to account for the discrepancies, it is not quite clear why titration of diluted media should overshoot the mark but it is evident that too great dilution in titration should be avoided.

AROMA-PRODUCING MICROÖRGANISMS

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Cultures of bacteria often produce specific odors of various degrees of intensity and thus enable us to recognize readily several species. Frequently the odor is unpleasantly noticeable in places where mass cultures of bacteria are kept.

The odor produced by the cultures may be a general group character or an individual specific characteristic. The typical putrefactive bacteria can for example, be recognized by their specific odor, while cultures of yeasts, molds and actinomycetes are readily recognized by their peculiar aroma. Old cultures of *Erythrob. prodigiosus* produce a typical odor of trimethylamine, *B. fitzianus*, the odor of sweat (caproic acid ester), *B. odorificus*, the odor of rotted manure, etc. The same is true in respect to pathogenic bacteria: old cultures of *Mycob. tuberculosis* produce a specific odor, *Ps. pyocyanea*, a sweetish odor, resembling that of the lime-tree flowers, *M. tetragenes* and *M. pyogenes*—an odor partly resembling that of glue, etc.

Large numbers of similar examples can be cited, but these do not interest us at present. There is a special group of microorganisms, the representatives of which can be properly named "aroma-producing microorganisms," including those species which produce in culture a strong ethereal aroma of varying intensity, reminding one of the odor of fruits: strawberry, pineapple, apple, pear, muskmelon, etc. Here belong also those microorganisms, which give the "characteristic odor" to various products used for food and beverage, such as milk, cheeses, butter, wine, etc. The names of the species belonging to this group are correspondingly formed by the addition of epithets: *aromati-*

cus, *aromafaciens*, *odorus*, *odoratus*, *esterifaciens*, *fragi*, *fragariae*, *nobilis*, etc., and often different authors describe different species under the same name. There are in the literature three different *Bact. aromaticum*—those of Beijerinck, Pammel and van der Leek, and three *Bact. odoratum*—of Henrici, Burri and Weiss.

Aroma-producing microorganisms are quite abundant in nature; they are found in the soil, water, hay, in various plant infusions, in milk, etc. Several species have been isolated from manure and human excreta, in one case, that of cholera (*Bact. praepollens* Maaszen). The accumulation of organic acids and alcohols in the culture, mutually combining *in statu nascendi* and forming complex esters, is the cause of formation of the complex ester odor. The esters of acetic, butyric and valerianic acids are those most often produced.

The property of forming pleasant aromatic products represents a changeable character, which is more or less rapidly lost on artificial cultivation. To regain the lost characteristic, the culture is transferred upon the corresponding natural substratum: bacteria forming a strawberry aroma,—on a decoction of strawberry leaves, bacteria with a pineapple aroma—upon a medium of pineapple leaves, bacteria with a radish-like odor—upon a medium of yellow radishes.

It has been further noticed that the same culture often changes its original pure fruity odor into an unpleasant odor. This can be explained in two different ways. We know that certain products of putrefaction, such as indol, give, in low concentrations, a very pleasant aroma. The presence of some indol in mixture with the ethereal oil of jasmine explains the wonderful aroma of this plant. In larger concentrations, indol possesses however a repulsive odor. The original pleasant odor in cultures of aroma-producing microorganisms may thus change into an unpleasant odor depending upon the concentration of aromatic substances.

But it is also possible that there is originally formed in the culture a pleasant aroma, which is later decomposed with the formation of substances of different odors, sometimes unpleasant ones. This is true of several aroma producing bacteria which

develop first in milk a fruity odor and later the characteristic sharp aroma of Swiss cheese. In some cases the reverse may take place: at first bad-smelling products are formed, later changing to aromatic products. This is true of *B. esterificans* which according to Maaszen develops in meat-pepton bouillon at first a strong odor of mercaptan, which later changes to a pineapple odor.

The group of aroma-producing microörganisms consists of representatives of various biological families. The most important of these are:

1. *Yeasts*. Aroma formation is the property of many cultivated and wild yeasts, especially those belonging to the genera *Mycoderma*, *Pichia*, *Willia* and partly *Torula*. These yeasts form on the surface of the nutritive substratum a dry pellicle or a mouldy growth, which produces a pleasant odor of complex esters of various flavors. However, an excessive development of pellicle-forming yeasts on the grape may give it too sharp an aroma, which spoils the fine bouquet of old wines.

A good medium for the development of pellicle-forming yeasts consists, according to Beijerinck, of a solution of ammonium acetate with some potassium phosphate. Lindner incubated at room temperature for this purpose moistened grains of barley placed in a large tube loosely plugged at both ends with cotton. The colonies of pellicle-forming yeasts developed on the surface of the grains in the form of beautiful rosettes, producing a fruity odor.

Discoveries of aromatic yeasts have been made at various times. Kayser separated in 1891 from pineapple juice a species of yeast producing on artificial media the pineapple aroma. Adametz and Wilckens reported in 1892 a series of experiments with a lactic yeast isolated by Adametz, *Sacch. lactis*, which forms, from lactose, ethyl alcohol, acetic acid and a corresponding complex ester. This species gave to butter a very pleasant aroma. Lafar and Will isolated from beer, in 1893 and 1899, pellicle-forming yeasts with fruity aromas. Similar discoveries were made later by various authors (Lindner, Klöcker, etc.).

Yeasts producing in culture a pear and pineapple-like odor

were found in 1902 by Bail on rotting leaves of rhubarb. A year later Mazé isolated from various cheeses yeasts fermenting lactose with the formation of a cheesy odor. Madame Bachinskaia-Raichenko isolated from Kumiss in 1911 two forms of yeasts which produce a pleasant wine aroma on malt and Kumiss media. The aroma producing property was lost on continued cultivation upon laboratory media.

In 1914, Edwards isolated 3 species of yeasts from North American cheese which produce on malt media a pineapple and strawberry odor.

The above references, which could be greatly increased, show that pellicle-forming yeasts are widely distributed in nature and that they may play an important part in the production of aroma in various products.

2. *Acetic acid bacteria*. On oxidizing ethyl alcohol to acetic acid these bacteria form as a by-product the ester of ethyl acetate. In factories where vinegar is obtained not from alcohol but from wine, as by the Orleans process, there are formed in addition to the above ester other aromatic substances, giving to the product a complex aroma much valued on the market.

3. *Lactic acid bacteria*. The aromatic bacteria belonging to this group play an important rôle in the bacteriology of milk and milk products, being among the causes of the specific aroma of milk products. Of the lactic acid bacteria the most energetic ester forming species are: the small streptococcus of Hagenberg and Weigmann, *Bac.* No. 18 Storch, *Lactobacillus lactis-acidi* Leichmann, *Bact. acidi-lactici* Hüppe, *B. K.* Weigmann, etc. However, several races of bacteria, bringing about a natural souring of milk, like *Lactob. lactis-acidi* and *Streptococcus Hollandicus* possess the same property of aroma formation, giving to milk and milk products a pleasant complex ester odor.

4. *Butyric acid bacteria*. The phenomenon of ester formation is especially noticeable in mixed cultures. Winogradski used such cultures for the growth of the anaerobic nitrogen-fixing organism *Clostridium Pasteurianum* belonging to the butyric acid bacteria. This species rapidly develops, in the presence of

oxygen, in a thin layer of liquid, due to a symbiotic development with aerobic species; the cultures thereby produced give off a complex-ester aroma resembling the odor of apples.

5. *Putrefactive bacteria*. The decomposition of proteins by means of bacteria is usually accompanied by the formation of evil smelling products of putrefactive decomposition. However, as Nencki properly pointed out in 1889, to characterize the action of the protein organisms it is not sufficient to know whether the odor of the products of decomposition is bad or good, but it is important to know the nature and degree of decomposition. Those species which decompose proteins with the formation of pleasant odors will therefore also belong to the putrefactive organisms. Numerous such microörganisms have been described, and we will later discuss in detail several of them. Species that play an important part in the ripening of cheese seem to belong here; the casein of the milk is decomposed with the formation of products of decomposition giving out a specific aroma.

6. *Pathogenic bacteria*. Cultures of some pathogenic bacteria, such as those of *Mycob. tuberculosis* and *Ps. pyocyanea* above mentioned, produce a pleasant aroma.

7. *Mold fungi*. Lindner isolated in 1889 a fungus *Sachsia suaveolens*, which occupies an intermediate place between yeasts and molds. It produces fermentation of malt liquids with the formation of a complex ester odor, resembling the odor of wine. This was the reason why Lindner called the fungus "Weinbouquet Schimmelpilz." An attempt was made to use this species technically to give a bouquet to non-alcoholic beverages. Mirsch and Eberhard took out a patent for the treatment of apple juice for ten days at 15 to 20°C. with this species till the surface of the liquid was covered with a pellicle of the mold and fermentation began. The liquid was then filtered and saturated with CO₂, producing a pleasant beverage with an aroma of Mosel-wine.

Several species of *Oidium lactis* impart to butter a nutty odor and a taste of mushrooms, while others give the odor of honey. The *Oidium suaveolens* isolated from water by Krzemeck develops a fruity aroma on malt media and on beer must. When growing upon sugar media it produces traces of ethyl alcohol and a small quantity of citric and malic acids.

Schnell isolated in 1912, from milk and milk products, several *Oidia* imparting to milk especially under acid conditions a more or less sharp odor and the taste of cheese. The author believes that they are normal agents in the ripening of cheese.

Several representatives of the genera *Penicillium*, *Mucor*, *Actinomyces*, etc. also produce a fruity odor, like *Penicillium aromaticum casei* I and II.

Kayser in 1891 and Went in 1893 isolated molds producing a pineapple odor. The latter isolated from Javan cane the fungus *Thielaviopsis aethaceticus*, which produces a pineapple odor in the presence of dextrin, sucrose and glucose. The fungus is a plant pathogen. Several Mucorales, like *M. piriformis*, *M. javanicus*, *M. mucedo*, *M. racemosus*, etc., impart to butter an odor of bitter almonds.

After this general reference to the complexity of the group of aroma-producing microorganisms, we may describe a few species in detail adhering to a chronological order.

The first reference to the presence in milk of bacteria which produce a fruity odor was made by Beijerinck. Milk placed in a shallow layer and coagulated by the rennetic enzyme produced after some time at room temperature a pleasant fruity aroma. By diluting and plating out on solid media, he succeeded in isolating a species possessing definite proteolytic properties at the same time producing an aroma; this species was called *Bact. aromaticum*. Weigmann isolated in 1890 a gelatin-liquefying, spore forming bacillus, *Aromabacillus Weigmanni*, imparting a fruity odor to milk. The same year Sclavo and Gosio described an aerobic spore-forming species, *B. suaveolens*, first hydrolyzing starch with the formation of dextrin and glucose, then decomposing it with the formation of esters of butyric and acetic acids. The same odor is produced by the growth of the organism in milk, meat extract and infusions of hay, straw and beet sugar. This species liquefies gelatin energetically.

Tatarov, in a Dissertation (Dorpat) on "Die Dorpater Wasserbakterien," described a weakly motile (one polar flagellum) bacillus ($1.5 \times 3.5-5 \mu$) with a round spore, isolated from well water, *B. crassus-aromaticus* Tatarov (= *Pseudomonas aromatica*

Migula). This species forms on meat pepton agar a white, moist growth, with the development of a fruity odor. Gelatin is liquefied in a funnel-shaped manner giving a dirty deposit at the bottom.

In 1894, Henrici isolated from cheese several species giving a complex ester odor of various shades, gradually changing into a cheesy odor: a facultative anaerobic form, a motile bacillus ($0.6 \times 0.3 \mu$), allied to *Bact. aromaticum*, liquefying gelatin, *B. odor*, forming a honey-like growth on meat pepton agar; *M. odor* and *adoratus* (1.8μ in diameter), etc.

The practical dairymen became greatly interested in the non-spore forming, non-motile bacillus ($0.7 \times 1.1 \mu$) often united in pairs and isolated by Conn in 1895 from South American (Uruguay) milk. This form is known as *Bacillus* No. 41 Conn and belongs to the *coli-aerogenes* group. Meat pepton bouillon is made turbid with the formation of a surface pellicle. On meat pepton gelatin, it grows better in the streak than on the surface. A shining white growth is formed on meat pepton agar. On inoculating milk with this species the acidity is first increased and a strong complex-ester odor is produced. Casein is then peptonized and the reaction becomes alkaline. The milk turns brownish and the aroma gradually becomes cheesy. When winter butter is inoculated with this species, it acquires the aroma of June butter, the "grass flavor," so highly prized on the market. However, not all investigators have obtained the same favorable results in the application of this species to practical butter making. This is probably due to the degeneration of the microbe and the loss of aroma-producing properties.

Pammel described in the same year a facultative anaerobic, motile, non-spore forming bacillus ($0.3\text{--}0.45 \times 0.9\text{--}1.2 \mu$) *Bact. aromaticum*, often united to form short chains, liquefying gelatin in a funnel-shaped manner and coagulating milk. Meat pepton bouillon is clouded with the formation of a yellow sediment. Growth on agar is at first white, then gray and finally brown. Glucose and sucrose are fermented with the formation of gas. In milk at first a pleasant aromatic odor ("butyl odor"), is produced which gradually changes to a stronger and distinctly

cheesy one, reminding one of the odor of Limburger cheese. Milk is coagulated and the coagulum is then gradually dissolved.

Bact. butyriaromafaciens isolated in 1897 by Keith also belongs to the acid-forming and gelatin-liquefying species; this is a non-motile, non-spore forming bacillus ($0.5 \times 1 \mu$) imparting to the milk a specific acid odor of butter. Milk is not coagulated. Bouillon is clouded with the formation of a sediment. An abundant white growth is formed on agar. Groups of two cells are often found in culture.

Burri isolated the same year, from 6 samples of Emmenthal cheese, the same species—a facultative anaerobic, motile bacillus ($1.3\text{--}1.5 \times 3\text{--}6 \mu$) with oval spores, named by Lehmann and Neumann *B. bernensis* and by Matsuschita *B. odoratus*. This species inoculated into agar forms fungus-like branching colonies. Gelatin is slowly liquefied in funnel shape. Bouillon becomes rapidly turbid with the formation of a pellicle on the surface and sediment on the bottom. Milk is coagulated in twenty-four hours at 30° . The coagulated casein is gradually dissolved, and the liquid becomes yellowish-brown, with the odor of Emmenthal cheese. The same odor is found to some extent in meat pepton media. Eckles isolated in 1898 from milk a non-motile aroma-forming bacillus (about 1.3μ long) *Bac.* No. 13 Eckles, related to *Bacillus* No. 41 Conn.

In an extensive work published in 1899, Maaszen described 4 species, the cultures of which produce aromatic odors:

1. *Bact. esterificans Stralauense* isolated from the water of the river Spree (near Berlin). This is a rather large, motile, non-spore forming bacillus, allied to the intestinal bacteria. It produces a slight ester-odor on meat pepton media, rapidly lost on further transfer.

2. *B. esterificans* isolated from putrefactive litmus solution, a facultative anaerobic, peritrichic bacillus ($0.5\text{--}0.6 \times 1.4\text{--}5.2 \mu$) with a large oval spore ($1.2\text{--}1.4 \times 2.7\text{--}3.2 \mu$). It produces in meat pepton media the odor of pineapple or fresh apples. Gelatin is not liquefied, with the formation along the streak of a granular white thread. On meat pepton bouillon at first a strong odor of mercaptan is formed and later a pineapple odor. The bouillon

is made very turbid with the formation of an abundant grayish-white deposit on the bottom. Milk is slowly coagulated (negative according to Husz) with the formation of a characteristic pineapple odor. The aroma-producing property is not lost by this species for a year.

3. *Bact. esterificans fluorescens* was found by Maaszen on grains, and on rotting plants in river water. This is a small, non-spore forming motile bacillus, producing in cultures a fruity odor, gradually changing to that of trimethylamine. Gelatin is not liquefied.

4. *Bact. praepollens* was isolated from the sweat of one sick with cholera. This is a non-spore forming non-motile bacillus, rapidly liquefying meat pepton, gelatin, blood serum and milk casein. It decomposes urea rapidly and can reduce nitrates. In the decomposition of pepton and growth on milk, there is produced an odor of amylvalerianic ester, especially in the presence of abundant aeration. Later, the odor loses its purity and gradually changes to one of ammonia and cheese. The property of aroma formation is kept for years. Carbohydrates are decomposed without the formation of gaseous products.

In 1899, Weiss isolated from turnips an aroma-forming bacillus, *Bact. odoratum*. In 1900, Reinmann isolated from bitter butter a motile bacterium belonging to the group of intestinal bacilli, or soil bacilli. On inoculating fresh butter with this species, a strawberry aroma is soon produced. The following year Glage isolated from common manure a short bacillus related to the intestinal bacteria but liquefying gelatin with a fruity odor.

Rullmann isolated in 1902 from milk a spore-forming, non-motile, fluorescent species, producing in cultures a fruity odor. Size $0.4 \times 1.5 \mu$. Spores-terminal. Gelatin is not liquefied. It belongs to the facultative anaerobes.

The same year (1902) appeared the work of Grimm, Eichholz and Grüber. The first isolated from milk a very motile, peritrichic bacillus ($0.8 \times 3.5 \mu$) *B. aromaticus lactis*, belonging to the facultative anaerobes. This bacillus forms in culture a great deal of slime and produces a complex ester odor, gradually changing to a cheesy one. A dirty-gray slime appears on meat

pepton agar. Gelatin is not liquefied, with the formation of pearl-like growth along the streak. Milk becomes slightly sour but is not coagulated; the milk gradually becomes brownish and a strong fruity odor develops due to the formation of ethyl lactate. After four to five weeks, the milk is coagulated with the formation of a cheesy odor. The coagulated casein is weakly peptonized.

Eichholz isolated from milk a monotrichic bacillus ($1.05 \times 1.75\text{--}2.10 \mu$), allied to the intestinal groups *Bact. fragi* (*Pseudomonas fragi*), producing on meat pepton agar at first a strawberry odor, then that of methylamine. The colonies of this species on lactose-gelatin are characteristic; showing a granular central portion with scallop-like edges in the form of a rosette. Gelatin is not liquefied. Milk is not coagulated, the reaction changes to alkaline and a rotting odor is formed.

Grüber isolated from river water a motile, non-spore forming bacillus *Pseudomonas fragariae* I., which produces a fluorescence in media. Bouillon is made turbid with a slimy sediment. The reaction becomes distinctly alkaline. Gelatin is not liquefied, growth is on the surface and not along the stab. On meat pepton media, the odor is at first that of strawberry and pineapple, in time changing into the ammoniacal odor of decomposing urea. A peculiar odor and bitter taste is imparted to milk. In a month the milk becomes brownish. Sterilized milk inoculated with this species produces after some time a strawberry odor.

In 1904, Severin isolated from cream a motile non-spore forming bacillus *Bact. aromaticus butyri*, producing on meat pepton media at first a fruity odor, then a rotting ammoniacal odor. Gelatin is not liquefied and milk is not coagulated. The fruity aroma is produced on milk only when lactic acid bacteria are also present, and is especially prominent during the period previous to milk coagulation. When added to fresh butter, this species imparts to it in a month a fruity aroma. The aroma producing property is not lost in eight months.

Gaethgen isolated in 1905 from secretions of individuals suspected of typhus, a non-spore forming, weakly motile bacillus, producing a strong fruity odor in milk, *Bact. flavo-aromaticum*,

forming yellow colored colonies. By its culture and odor formation in culture, this species is related to *B. crassus aromaticus* Tatarov. Gelatin is not liquefied.

Grüber isolated the same year, from pasteurized milk, a motile bacillus, *Pseudomonas fragariae* II, producing a marked odor of strawberries on meat pepton media, especially gelatin, which is weakly liquefied on the surface.

Beck described in 1906 an acid-forming *Micrococcus esterificans*, developing a complex ester fruity odor. This species acts very weakly on proteins.

Huss described in 1907 two species producing a fruity aroma: *Bact. esterificans* and *Pseudomonas Trifolii*. The first species was first isolated by Maaszen, but Huss described it in detail. The second was found on clover. This is a monotrichic, non-spore forming bacillus ($0.5-0.7 \times 0.75-2.1 \mu$), growing on agar in the form of a transparent gray-yellow growth, reminding a layer of vaselin. It liquefies gelatin and decomposes proteins, with the formation of H_2S . On media containing carbohydrates and proteins, the carbohydrates are first decomposed and the liquid changes from acid to alkaline. Milk is coagulated in two weeks by means of rennet, with an odor of fresh hay. The coagulated casein gradually dissolves, and the milk develops a bitter taste. In another article published the same year, Huss described a motile non-spore forming bacillus ($0.6 \times 1-4 \mu$), *Pseudomonas fragaroidea*, developing in cultures first a strawberry, then an ammoniacal odor. On meat pepton bouillon and agar, fluorescence and a strong alkaline reaction are produced. Gelatin is rapidly liquefied. Milk is coagulated without change in reaction and the coagulum is gradually dissolved. The original strawberry odor changes to pineapple.

In 1907 there also appeared an article by van der Leek, describing two microbes with a fruity odor:

1. *Bact. aromaticum*, a very motile bacillus ($0.8 \times 2.5-3.5 \mu$); allied to *Bac.* No. 41 Conn. It forms yellowish slimy colonies on gelatin, slowly liquefying it around the colony with the formation of an odor. A dirty-white slimy growth on agar. Under the influence of this microbe the milk obtains a peculiar, acidic

aroma with very weak acidity; it coagulates very slowly and turns gradually brown. Of the carbohydrates, only glucose is utilized. The optimum temperature for growth is 23°C.

2. *Bact. acido-aromaticum*, a non-motile and gelatin non-liquefying bacillus of the same size as the previous form. It makes the milk quickly acid with the formation of a butyric acid odor.

For the accumulation in culture and separation of aroma-producing bacteria, van der Leek used the method first introduced by Beijerinck. Market milk is warmed to 30°C. and, adding some rennet, is poured in a thin layer into a Petri dish. The milk is coagulated at 20 to 23° and usually begins to produce in twenty-four hours a fruity odor. A gelatin medium prepared with milk whey is used for isolation.

Kurono isolated in 1911 two aroma-producing races of *B. butyricus* from the rice-wine "Saké-Moromi." By fermenting glucose with these species an ethyl butyric ester is formed.

The above list of aroma-producing microbes is far from complete and cannot even pretend to be, particularly since many sources remained unattainable for the author, especially during the last few years. Nevertheless, even on the basis of the material presented, a definite idea can be obtained of the great variety of this group.

Among the sources from which the organisms were isolated, we find milk products, water, excreta, etc. However, the source of isolation cannot serve any purpose for explaining the peculiarities of the isolated microbe. This explanation can be given only in special cases. For example, the microbes isolated from milk develop in artificial cultures products having a cheesy odor.

The same lack of uniformity is also found in the morphological characters of the organisms. We find here cocci, short and long bacilli, spore forms and non-spore forms, motile and non-motile forms. A large number of the species belong to the coli-aerogenes group or are closely related to them.

There is also a great difference between the various species in the type of odor and constancy of aroma-production, which is rapidly lost for many species in cultivation on artificial media.

Only a few general features are recognized here. For example, bacteria originally producing a very fine strawberry aroma later usually change it into a sharp ammoniacal odor, that of putrefying urine or a specific putrefactive odor, as in the case of *Bact. fragi* Eichholz, *Pseudomonas fragariae* I Gruber and *Pseudomonas fragaroidae* Huss.

Attention has been already called to the variety of biochemical characteristics of the aroma-producing microbes. Attention must be drawn here to the fact that some species were described so superficially, that it is impossible to get a clear idea of the chemistry of their processes. The great number of organisms capable of decomposing proteins is marked: *Bact. aromaticum* Beijerinck, *B. crassus aromaticus*, *B. odorus*, *Bac.* No. 41, *B. bernensis*, *Bact. praepollens*, *Bact. fragi*, *Bact. fragariae*, etc.

This makes possible the hypothesis that the main cause of aroma-formation in wine, milk and milk products is the production of odoriferous products from the decomposition of proteins.

This group of microörganisms has been attracting, for the last few years, more and more attention since to the species belonging here is ascribed an important part in the natural production of aroma in various food products, especially milk products—butter, cheese, curdled milk, milk, etc. Their rôle in imparting a bouquet to various wines of grape, berries and fruit, as well as beer is also possible.

This investigation is devoted to the description of several new species of aroma-producing microbes.

The question of aroma-producing bacteria came up twice, in my laboratory experience, and both times accidentally, in 1897 and 1915.

In the spring of 1897, when working with cellulose-decomposing bacteria, an experiment was started dealing with the decomposition of pieces of raw potato in an open beaker filled with tap water. An active fermentation took place accompanied by gas production, and the potato gradually decomposed and became transformed into a starchy mass. The turbid liquid above the rotted material gave out a repulsive odor. Several dilutions

were made from this upon meat pepton agar. After some time at 35°C. the cultures in the Petri dishes began to produce a pleasant fruity aroma resembling the odor of apples. By transferring the minute colonies developing upon the agar, a pure culture was obtained of the organism causing this odor. It proved to be a non-characteristic, short, non-spore-forming bacillus, belonging to the protein decomposing bacteria and entirely inactive in regard to the carbohydrates. After a few preliminary experiments the work had to be stopped due to my leaving Petrograd for the summer. When the work was resumed it was found, to my great regret, that the culture had died during the summer and could not be revived. Attempts to reisolate the culture using the previous method proved to be also in vain.

Eighteen years later, in the fall of 1915, I succeeded in isolating the organism again. It is known that in the preparation of anti-rabies virus the brain of the rabbit used as virus is first tested for sterility. For that purpose a piece of it is placed in a tube with sterile bouillon and the latter is incubated at 37°C. If the bouillon becomes turbid and bacteria multiply in it, the brain tissue cannot be used. Such a case, which is a comparatively rare one, took place in August, 1915 in the Inoculation Department of the Institute of Experimental Medicine, the turbid bouillon producing a fruity aroma. The tube with the bouillon was kindly given to me by Dr. R. G. Pirone. Most unexpectedly the same organisms which I had possessed many years ago developed in the tube. Again, I succeeded in obtaining rapidly a pure culture without any difficulties by merely diluting on meat pepton agar.

Before describing this species I must remark that its original habitat remains unknown to me, since both times the organism was found by accident, and attempts to obtain it from raw materials gave no results. But judging by the repeated occurrence of this organism one may conclude that this species is widely distributed in nature.

Like the majority of described bacteria producing a fruity odor, the species that I have isolated belongs to the small, non-spore forming bacteria, closely related in appearance to the intestinal bacillus. Its dimensions are $0.5 \times 1-3 \mu$. By ex-

aming an emulsion of live bacteria in a drop of water, no active motility was observed. Attempts to demonstrate flagella by means of various processes of staining also remained unsuccessful. This species does not produce any slimy capsules or zooglea formations and is, in general, very poor in distinguishing characteristics. Yet since, by biochemical characteristics, it cannot be identified with any of the previously described forms, it is suggested that it be recognized as an independent form, calling it, after its most distinguished character, that of forming aromatic products—*Bact. esteroaromaticum*. It belongs to the facultative anaerobes, growing better, however, with the admission of air.

This bacterium possesses a distinct reducing action. A bouillon culture in a tube colored with a solution of methylene blue is decolorized within one minute. By shaking up the liquid, the color again returns. A suspension of bacteria obtained from an agar slant in water does not possess this reducing property. This leads us to think that the reduction is a property of some products of metabolism dissolving in the bouillon. The decolorization of indigo carmin and neutral red took place just as rapidly.

Arsenates added in concentrations of 0.001 to 0.02 per cent to the medium are not reduced: no garlic odor is produced by the cultures. By inoculating a slant of meat pepton agar to which 2 drops of a 2 per cent potassium tellurate¹ is added, the bacterial growth became blackened on the second day, especially near the water of condensation.

Meat pepton bouillon becomes rapidly turbid, especially in the upper layers. At 25° there is produced on the third day a faint fruity aroma, gradually becoming more pronounced but losing in purity. This odor with a definite flavor of esters of valerianic acid, reminds one of the aroma of apples, changing in degree depending on the composition of the medium and conditions of cultivation. By inoculating meat pepton gelatin, cup-like liquefaction begins at room temperature on the second day. In a week, the *depth* of the liquefied portion is 1.5 cm. and the culture produces an odor resembling that of musk-melons.

¹ Sodium tellurate commonly used could not be obtained in the laboratory.

On meat pepton agar at 35° there is produced a fruity aroma on the second day and the medium becomes slightly phosphorescent. The colonies are of a yellow-brown color with slight festoon-like edges. On agar containing 1 per cent glucose the odor is weaker and not so pure as on plain agar. The addition of a few drops of ethyl alcohol to meat pepton agar notably increases aroma-formation. Fluorescence is more pronounced on this medium. Meat pepton agar with 6 per cent glycerol is less adapted to the development of *Bact. esteroaromaticum*, although a strong aroma is produced with a flavor of the musk-melon odor.

Heyden's agar is little adapted to the development of the organism, but even on this medium an aroma is produced. Only a weak growth without any odor formation takes place on malt agar.

The growth of the microbe in milk is characteristic. During the first few days the milk produces the repellent odor of a lard candle, due to the decomposition of the fat. The casein dissolves gradually, starting from the surface, the milk becoming light brown. Milk free from fat produces from the beginning a pleasant aroma, recalling that of valerianic ester. This odor changes finally to the characteristic aroma of old cheese; especially when the milk is made slightly acid. The addition of 0.5 to 1 per cent pepton stimulates the growth of the organism and its aroma formation.

Growth on potato is abundant, but the odor is unpleasant. A three per cent pepton water becomes slightly turbid with the development of a light fruity odor.

A strong and pure aroma of a complex ester is produced, on the third day, on a medium of the following composition:

Tap water.....	1000 cc.
Pepton.....	5-10 gm.
Potassium phosphate.....	1 gm.
Agar-agar.....	20 gm.

especially if a few drops of ethyl alcohol are added.

A streak on an ox-serum slant shows on the second day, liquefaction along the streak, which becomes deeper, while the lique-

fied serum flows down in the form of a brown mass. All the serum is liquefied on the fifth day with the production of a fruity aroma.

On the basis of the above data one may conclude that *Bact. esteroaromaticum* belongs to the protein decomposing organisms, growing best on pepton media. The addition of propyl alcohol to the medium has no influence on the strength or quality of the odor. Amyl alcohol possesses by itself such a strong aroma that it represses the fruity aroma of the culture. The addition of mannitol has a depressing effect upon aroma formation in pepton media. The increase in concentration of pepton to 2 per cent stimulates the formation of aromatic substances.

A fair growth and a pleasant fruity aroma with a peculiar flavor is found in media containing egg or blood albumen as in the case of the following medium:

Distilled water.....	1000 cc.
Egg albumen (Kahlbaum).....	50 gm.
Potassium phosphate.....	1 gm.
Magnesium sulfate.....	0.5 gm.
Sodium chloride.....	0.1 gm.

The egg albumen is first dissolved in a warm solution, and the liquid is placed in 50 cc. portions in Winogradski flasks. After sterilization the albumen solution is precipitated in the form of a solid white mass. It begins to liquefy on the second day, and in a week all the albumen is dissolved. The culture produces a strong aroma with a characteristic flavor. On gelatin, wool, nutrose, aleuron, gliadin and keratin, no growth or aroma formation takes place. *Bact. esteroaromaticum* acts upon casein (nach Hammersten-Merck) only in the presence of soda: 0.1 gram per 1 gram of casein. Under these conditions, casein is readily dissolved with the formation of the complex ester aroma.

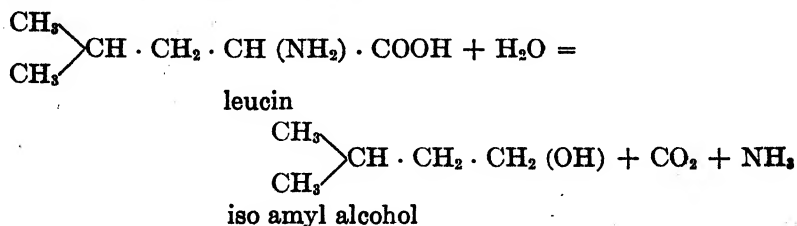
As pointed out above, milk casein as well as casein precipitated by rennet is rapidly dissolved. But the casein from sour milk is not acted upon due to the reaction of the medium.

The organism develops readily with the formation of a fruity aroma on "vegetable casein" or legumin. The organism acts energetically on fish protein prepared by Prof. M. D. Ilün and

kindly placed at our disposal for investigation. The protein mass, grayish in color, was faintly acid and, for the favorable action of the microbe, it had to be neutralized (1 cc. of a 5 per cent solution of soda to 5 gram of protein). In a few days, after inoculation at 30°, peptonization of the mass and odor formation took place. Of the amino acids (and acid amides), alanin, asparagin and urea are unsuitable for the development of *Bact. esteroaromaticum* alone and in the presence of carbohydrates. Weak growth takes place upon 1 per cent solution of leucin and α -amino-isocaproic acid. The addition of leucin to meat pepton bouillon or to pepton solution stimulated the growth of the organism and the formation of valerianic ester, especially in the presence of ethyl alcohol.

The mechanism of ester formation and the intermediary stages through which the protein passes when it is decomposed by the organism are of great interest. Unfortunately, one can answer the question only by indirect considerations. In view of the fact that in the decomposition of proteins by *Bact. esteroaromaticum* esters of valerianic acid are formed, it appears that proteins are decomposed with the splitting of leucin or iso-leucin and the latter is further decomposed with the formation of valerianic acid, amyl alcohol and compounds of the ester type, as iso-amyl-valerianic ester (found by Maaszen in the decomposition of proteins by *Bact. praepollens*). The esters are probably formed by the interaction of the alcohols and acids.

The formation of optically inactive iso-amyl alcohol from *L*-leucin takes place according to the equation:



Iso-leucin is transformed in an analogous way into optically active *d*-amyl alcohol, by the splitting off of CO₂. This transformation has been observed by F. Ehrlich in the decomposition of leucin by yeasts and by Pringsheim for molds.

This method is the more probable, since in the decomposition of proteins by *Bact. esteroaromaticum* an odor of apples is produced, particularly strong in the presence of ethyl alcohol, an odor, as is well known, determined by the ester of iso-amyl-isovalerianic acid. This ester mixed with 5 to 6 parts of ethyl alcohol is used for obtaining the artificial "apple ester."

In old cultures the ester aroma passes gradually into a cheesy odor, especially noticeable in milk. It is known that one of the causes which determine the odor of old ("rotten") cheese is the presence among the products of decomposition of isovalerianic acid. As will be seen later, there are direct indications of the formation of valerianic acid in the decomposition of proteins by our microbe. All these considerations make us recognize as entirely probable the supposition concerning the mechanism of decomposition of proteins by *Bact. esteroaromaticum* with the formation as intermediate products of leucin and iso-leucin and from these, various compounds of valerianic acid.

As seen above, free ammonia is formed in the decomposition of leucin. Considerable quantities of ammonia could be demonstrated in the cultures from the decomposition of proteins. The ammonia is determined qualitatively and quantitatively by distilling with magnesium oxide and titration of the N_{10}^{N} sulfuric acid.

Among the products of decomposition of the proteins, the presence of H_2S was demonstrated. A piece of paper moistened with lead acetate solution and placed above the culture of *Bact. esteroaromaticum* darkened on the second day. On agar with lead acetate, the colonies became brown.

Neither indol nor tryptophane were demonstrated in two week old pepton cultures. Weyl's reaction for creatinin gave positive results.

To obtain a more definite measure of the degree of decomposition of different proteins by our organism the amino nitrogen was determined by the method of Sørensen. One hundred cubic centimeter portions of the mineral medium were placed in 5 Winogradski flasks and 1-gram portions of the following proteins were added to the different flasks: egg albumen (Kahlbaum),

blood albumen (Kahlbaum), pepton (Okunev), casein nach Hammarsten (Merck) and keratin-Zyzkia (Merck).

The flasks were kept at 30 to 31° for two weeks. The results are as follows:

KIND OF PROTEIN	DEVELOPMENT OF ORGANISM	DEGREE OF AROMA-FORMATION	AMINO-NITROGEN
			<i>mgm.</i>
Egg albumen.....	Very good*	Pure fruity aroma	47.6
Blood albumen.....	Very good*	Odor not so pure	47.6
Peptone.....	Very good*	Odor of ammonia	43.4
Casein.....	No growth	No odor	5.6
Keratin.....	No growth	No odor	5.6

* Rapid peptonization.

In another experiment, 5 per cent egg albumen (Kahlbaum) was added to the mineral solution, and 50-cc. portions of the liquid containing 2.5 gram albumen each were placed in 20 Winogradski flasks. Three drops of a suspension of *Bact. estero-aromaticum* grown on agar were added to each flask.

On the second day, orange-yellow spots appeared on the surface of the coagulated albumen at the places of inoculation and the culture began to produce a fruity odor with the flavor of valerianic ester. The peptonization of the albumen was noticeable on the third day. The amino nitrogen was determined in one flask on the fourth day, according to Sørensen, and it was found to be 44.8 mgm. to 1 gram of albumen.

Since energetic peptonization was observed in all the flasks, it was thought of interest to determine the content of proteolytic enzyme. The determination was carried out according to the methods of Fuld-Gross and Fermi.² Two hundred cubic centimeters of water were added to the contents of one of the flasks to make a 1 per cent solution of the original protein. The turbid liquid was filtered through a paper filter and the filtrate distributed in 10 tubes in decreasing quantities (cubic cm.): 1, 0.5, 0.25, 0.125, etc., i.e., in decreasing geometrical ratio. By the

² Wohlgemuth, Grundriss der Fermentmethoden, 1913.

Fuld-Gross method, after one hour's action at 38°, a complete dissolution of the casein was obtained only in the first tube (1 cc.) and partial dissolution in the second, with complete liquefaction of the gelatin in the first tube according to the method of Fermi.

On the seventeenth day after inoculation, the contents of all the flasks were poured together for a general analysis. A microscopic examination of the liquid indicated that the culture remained uncontaminated. The thick yellowish-brown liquid possessed an apple aroma with a flavor partly of valerianic ester and partly of ammonia. The reaction of the liquid was distinctly alkaline. The following analysis were made: total nitrogen according to Kjeldahl, amino-nitrogen by the method of Sørensen, peptide nitrogen by the method of Henriques-Sørensen and ammoniacal nitrogen by the method of Spiro. In addition to this the following protein color reactions were made: biuret, Millon, Liebermann, Adamkiewicz, Keanto-protein, Molish-Udranovski, Pettenkoffer and lead acetate.

For analysis there were used: I, liquid of original culture; II, same liquid, made slightly acid with acetic acid and filtered after boiling; and III, second liquid boiled with animal charcoal.

All color reactions gave good results with the first liquid, but were weak with the second and negative with the third. The following table gives the various nitrogen determinations, on the basis of 1 gram original protein, in milligrams.

	I	II	III
Total-N.....	122.5	71.8	39.1
Amino-N.....	53.2	46.0	34.5
Peptide-N.....			34.5
Ammonia-N.....			1.18

The composition of the volatile fatty acids was determined, according to Duclaux, in a separate experiment. The liquid, slightly acidified by means of oxalic acid, was distilled three times with the following results:

FRACTION	$\frac{N}{20}$ NaOH	PER CENT	THEORETICAL AMOUNT OF MIXTURE 1 PART ACETIC AND 1 PART VALERIANIC ACIDS
	cc.		
1	11.2	18.8	
2	20.0	33.5	34.1
3	27.5	46.1	46.4
4	33.9	56.7	64.7
5	39.4	66.0	64.7
6	44.1	74.0	77.0
7	48.3	80.9	78.7
8	52.2	87.4	
9	55.8	93.4	
10	59.7	100.0	

The correlation is not very perfect, but the discrepancies among the figures can be explained by the admixture of other acids, to which indications seemed to point. For example, the solution of volatile acids gave the reaction with silver nitrate (admixture of formic acid).

Bact. esteroaromaticum does not attack carbohydrates, higher alcohols and salts of organic acids. Among the substances tested were: glucose, sucrose and lactose, dextrin, inulin, starch, gum-arabic, dulcitol, mannitol and ammonium tartrate. All these compounds were of no use for the cultivation of the organism in the absence of proteins.

It has been already stated that the organism hydrolyzes milk fat. It has the same action on fish fat added to meat pepton agar. It does not act on sunflower oil under the same conditions.

Tests for denitrification gave negative results: not only was no free nitrogen produced, but there was even no reduction of nitrates to nitrites.

On the basis of the above data, we may conclude that this organism belongs to the typical protein bacteria, readily growing on media with egg and blood albumen, on casein (in the presence of soda), pepton, etc. All these substances form sources of both nitrogen and carbon. The property of aroma formation distinguishes our organism from the morphologically related common species; as to the aroma producing forms, none is identical with

it. It is most closely related to *Bact. praepollens* Maaszen, but the latter is capable of denitrifying nitrates and decomposing urea.

Some brief observations during this work indicated the fact that the degree and character of aroma formation may change in the presence of other species. In one case the fruity odor was markedly increased in symbiosis of *Bact. esteroaromaticum* with a large, non-spore forming bacillus which was isolated from mud and fermented various carbohydrates energetically. The influence of this species was particularly marked in sugar media. The co-action with other species has not been studied in greater detail, although this question is of great interest.

The idea naturally suggested itself of utilizing the isolated species for practical purposes, namely, to give an aroma to various milk products, particularly cheese. Until now the application of aroma-forming bacteria to practical purposes has been difficult due to the fact that they rapidly lost the property of producing aromatic products when cultivated in the laboratory on artificial media. This physiological degeneration doubtless explains the lack of correlation between the results obtained by various authors for the same species. While *Bacillus* No. 41 gave excellent results in the hands of Conn and other American investigators, Farrington and Russell could not get any results at all. Another example is *B. nobilis* isolated by Adametz and Kleck and recommended by them for increasing the aroma of cheese. Dried preparations of this culture were placed on the market under the name of "Tyrogen." However, the cheese prepared by Freudenreich and Troili-Peterson, by means of this culture, was of a poorer quality than the control, doubtless due to the degeneration of the species and loss of original properties.

In the light of these and similar facts, the ability of *Bact. esteroaromaticum* to preserve for several years, without noticeable weakening, the property of aroma formation is of special importance. It is three and one-half years since the microbe was isolated and it still preserves, almost completely, its properties.

The capacity of *Bact. esteroaromaticum* to hydrolyze fats made us give up early the idea of adding aroma to butter. However,

various facts were in favor of its application to the ripening of cheeses, especially those prepared from skimmed milk. One would think that, in addition to its adding a proper aroma to the cheese this species might partially hydrolyze the casein and impart the proper consistency to the milk, which is very important for heavy cheeses.

Unfortunately, the experiments performed up to the present did not give satisfactory results due to purely accidental causes.³ In The Nadezshdin Dairy School (Tambov Gov.) the culture was added 5-10 minutes before cooking and the chemical action of the organism did not become pronounced. Neither an aromatic nor a cheesy odor were produced. The prepared cheeses, Holland and Tilzit, were naturally only to a slight degree different from the controls. The results of the application of the culture of our organism in the Dairy Station in the Tuga Uyezd, Petrograd Gov., cannot be related as both the experimental and control cheeses were stolen from the cellar. The cheeses prepared in the third Station were forwarded to the address of A. A. Kalantar, but were requisitioned on the way and have not reached their destination. Unexpected results were obtained, according to C. A. Korolev, at the Lotoshin Experiment Station, where the experimental cheese swelled up and burst open. If this was due to gas formation, the organism in question is surely not the cause, since it produces no gas in culture. This phenomenon was not observed in the other stations.

It would be very desirable to repeat the experiments in the coming spring campaign of cheese making. The following experiments suggest themselves. Fresh milk is separated from fat and is abundantly inoculated with a culture of *Bact. esteromaticum*. The milk is kept at 30 to 35° for several periods to determine the optimum, at the end of which it is placed in the cellar for various periods. It seems that by the proper use of the culture the period of ripening could be greatly diminished without loss of flavor and plasticity.

³ The author takes here the opportunity of expressing his sincere thanks to the cooperation rendered by A. A. Kalantar, member of Scientific Committee of the previous Ministry of Agriculture; O. I. Ivashkevitsch, Director of the Nadezshdin Dairy School; and to C. A. Korolev, Director of the Moskau Bacteriologo-Agronomical Station.

It is possible that the organism will find application in the leather industry—in the process of tanning especially in softening the skins.

These are only possibilities, since under the present conditions it is difficult, almost impossible, to conduct large technical experiments.

I isolated another organism from milk in February, 1918, by the method of Beijerinck and van der Leek. One hundred cubic centimeters of good milk received from the Milk Section of the Central Food Department were placed in a Winogradski flask and coagulated by means of rennet (Lab powder 1 = 300,000 Witte). In twenty-four hours, at 18°C., the cultures began to produce a light strawberry aroma, becoming much stronger in another twenty-four hours. Dilutions were prepared from this culture using meat pepton agar containing 1 per cent glucose and a pure culture was easily obtained of the organism producing the same aroma. This proved to be a non-motile, non-spore forming, aerobic bacillus, $0.8 \times 1.2\text{--}2\mu$, with small capsules around the cells seen in the India-ink preparation.

Meat pepton bouillon becomes uniformly turbid on the second day, forming a small sediment and developing a light strawberry aroma. In a few days a pellicle is formed on the surface of the bouillon and the odor becomes sharper, gradually changing into an aroma recalling that of cheese.

On meat pepton agar, an abundant white slimy growth appears along the streak on the second day. The culture produces a slight strawberry odor, remaining practically unchanged for three weeks. Growth on agar containing sugar is abundant, but no odor is formed. The latter appears only after two weeks, recalling the aroma of cheese. Meat pepton gelatin is gradually liquefied producing a fruity aroma. On potatoes, there is at first a brownish-rosy growth practically without any odor, then the growth becomes of a more pronounced rose color, with the development of an excellent strawberry aroma.

The milk is coagulated in four days with a slight strawberry aroma. On titrating with $\frac{N}{10}$ sodium hydroxide, it gave on the

fifth day 21° Thörner. We can thus conclude that coagulation takes place not by means of an acid but by lab ferment.

This species belongs to the protein decomposing organisms, growing very well on solutions of pepton, egg albumen, casein and other proteins. In many cases, however, an impure fruity aroma develops with an admixture of other flavors and sometimes this is quite unpleasant. An odor of hay develops in solutions of serum albumen.

The experiments with this species were soon stopped, since the culture completely lost the property of aroma formation and all attempts to renew it ended fruitlessly.

It is known that pressed yeasts, when kept for a long time, begin to produce a sharp complex-ester odor. We observed also such an instance on bread Kvas kept for a long time, on the surface of which a "mold" appeared; and then began to produce a fruity aroma. Mrs. O. C. Manoilov and C. U. Buchbinder studying in my laboratory the question of bread fermentation isolated several species of pellicle yeasts from various materials: (1) from market bread Kvas, (2) from a sample of bread yeast received from Zarskoselski yesd, Petrograd. Gov., and (3) from bread yeast received from Lifland.

All these pellicle yeasts, little distinguished morphologically and belonging to the genus *Mycoderma*, possessed almost the same physiological properties. To grow well on sugar solutions, they required an abundance of oxygen. The cultures were usually kept on media of the following composition:

Tap water.....	1000 cc.
Glucose.....	100 gm.
Ammonium phosphate.....	2 gm.
Potassium chloride.....	0.1 gm.
Magnesium sulfate.....	0.1 gm.

The solution is placed in a thin layer in Winogradski flasks and after sterilization inoculated with pure cultures of mycodermae. A pellicle appeared usually in two days at 30 to 35° on the surface of the liquid and the culture produced a thin sourish aroma, reminding one of the odor of table wine. No fermentation took place. On microscopical examination the characteristic cells

of pellicle yeasts were found with two vacuoles and droplets of fat in the opposite ends. These yeasts did not grow in media with cane sugar. This is characteristic of pellicle yeasts. As is well known, this property suggested (Beijerinck) the purification of cane sugar from glucose. No growth took place in solutions of galactose, lactose, maltose, dextrin, starch, gum-arabic and mannitol.

The observation was made that the fruity aroma produced in media with glucose was increased on adding 1 to 2 per cent ethyl alcohol and a few drops of acetic acid. The fact that mycodermae cannot utilize pepton, asparagin and other organic nitrogen compounds as well as ammonium salts and also the need of large quantities of carbohydrates so that a good growth should take place indicate that the sources of ester formation are in this case not the proteins, as in the above described bacteria, but the carbohydrates. Alcohols and organic acids are produced in the decomposition of the carbohydrates and form, *in statu nascendi*, the complex compounds.

In conclusion, we may mention one aroma producing mold isolated from a moldy lemon. This mold belonging to the species *Penicillium*, produced a fruity aroma of a peculiar flavor, recalling that of a musk-melon, only on media of a certain definite composition, as in bean decoction (Mazé) to which 5 to 10 per cent of maltose is added. When maltose is replaced by starch or inulin, an unpleasant odor develops on the same medium. Finally, with some sugars, the odor is not produced at all.

A STUDY OF THE FORMATION OF GUM LEVAN FROM SUCROSE

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A large number of species of bacteria are known to produce gums from sugars. These may be classified into two main classes according to whether they form capsules, like the *Leuconostoc mesenteroides*, and *Semiclostridium commune*, or whether such capsules are absent, as in the case of Kramer's (1889) *Bacillus viscosus-sacchari*, Fritz Glaser's (1895) *Bacterium gelatinosum betae*, or Ritser's (1891) *Bacterium gummosum*. These gum-forming species of bacteria may be still further classified according to the type of sugars which they are capable of utilizing for gum production. There are those on the one hand which form gum from either sucrose or invert sugar, others only from sucrose; one has been described which forms it from sucrose or maltose, and another from sucrose or raffinose. The species of bacteria comprising these three classes are as follows:

- I. Gum formed either sucrose or invert sugar
Leuconostoc mesenteroides
Bacterium gelatinosum betae (Glaser)
- II. Gum formed only from sucrose
Bacterium gummosum (Ritser, 1891)
Bacillus gummosum (Happ, 1893)
Bacillus levaniiformans (Smith, 1901, 1902a)
The Ginger Beer plant of Ward's consisting of *Bacterium vermiforme*
and *Saccharomyces pyriformis*. (1899)
- III. Gum formed from sucrose or maltose
Micrococcus gummosus (Happ, 1893)
- IV. Gum formed only from sucrose or raffinose
Bacterium eucalypti (Smith, 1902b)

The species of bacteria of the first group are generally supposed to secrete invertase, and hence the gum that they form

is thought to be from the product of the inversion of the sucrose rather than from the sucrose itself. It is quite different with the second class, in which with the exception of the Ginger beer plant, no action of invertase is indicated. Its presence is inferred in the latter case only because of the fact that a true saccharomycete is involved in the transformation of the sucrose into gum.

In studying the gum-forming ability of the species of bacteria which he isolated from raw cane sugars, and which he named *Bacillus levaniiformans*, Greig Smith (1901, 1902 a) discovered that it produced this gum only from sucrose. Neither glucose nor levulose, nor any combination of these sugars, was suitable as material for the formation of gum by these organisms. He concluded therefore that gum levan could only be formed from glucose or levulose in their nascent condition, thus assuming the action of invertase, where its presence had never been proved. The proof or disproof of this theory seemed relatively simple to the writer, who several years ago carried out some experiments in which levan formation in the presence and absence of growing yeasts was compared (1911 a and b). The results of these experiments showed that the amount of gum levan formed from the mixed inoculation of yeast and bacteria was never more than one-half, and frequently only one-tenth of that formed by pure cultures of the bacteria. These results showed that the relationship between yeast and bacteria was not a symbiotic one, and suggested that the presence of invertase deprived the bacteria of a suitable form of sugar for gum production. The conditions of those experiments were not entirely free from criticism, and the conclusions drawn from them might be objected to on the possible grounds that the relationship between the yeast and the bacteria were competitive. The decreased production of levan might be regarded as due to the inhibiting effect of the yeast upon the development of the bacteria rather than to the transformation by the former of the sucrose into sugars that the bacteria could not utilize. In order to remove such objections it was decided to use invertase instead of inoculation with yeasts. The amount of invertase added was to be so regulated that the

bacteria would have a slowly diminishing supply of sucrose to act upon, or if, as has been claimed, nascent glucose or levulose is the source of the gum, the microorganisms would be provided with a steady supply of this material in quantities commensurate with their power of assimilation.

PLAN OF THE EXPERIMENTS

The culture solution used for testing the gum-forming power of the bacteria was that used by Greig Smith (1902 a). The formula was modified as regards its sucrose content, as we used a 20 per cent instead of a 10 per cent sucrose solution. This was necessary in order that the period of inversion might be sufficiently prolonged so that it might proceed throughout the entire incubation period. The formula was as follows:

	<i>per cent</i>
Sucrose.....	20
KCl.....	0.5
Na ₂ HPO ₄	0.2
Pepton.....	0.1

Two hundred cubic centimeter portions of this solution were poured into 300 cc. Erlenmeyer flasks, which were plugged with cotton and sterilized for thirty minutes on each of three successive days. The contents of the flasks were then inoculated with a pure culture of the levan-forming bacteria that had been isolated from a raw sugar. The inoculations were always made from forty-eight-hour cultures of the bacteria in the above solution. The invertase used in the experiments had been prepared by Kopeloff (1920) from Fleischmann's yeast. The invertase was added to the flasks with a sterile pipette, and they were then incubated for a period of ten days at 32°C. The analytical determinations were made as follows: The densities were determined by the Abbé refractometer, and expressed in degrees Brix. Sucrose was determined by the polariscope, the Clerget method of double polarization being used, and reducing sugars determined by the volumetric method of copper reduction, using Soxhlet's solution and Ross's (1912) method of testing for unreduced copper. For the determination of gum levan the

method of Kopeloff and Taggart (1920) was used. This method consists in calculating the levan present from the difference between the Clerget values by the acid and invertase method of inversion. The factor of 1.27 is used to convert this difference in values into the per cent of gum. This factor is derived from the change in specific rotation of gum levan from -40 to -92.5 when converted into fructose by hydrolysis with the acid used for inversion. This difference of -52.5 between the Clerget and the invertase values in the specific rotation of gum corre-

TABLE 1

The results from Experiment 1 in which invertase was used in the following amounts, 0.1, 0.25, 0.5, 0.75, and 1 cc.

SAMPLE	TOTAL SOLIDS BY RE-FRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
Inoculated, 1 cc. bacteria, 1 cc. invertase.....	23.19	-5.1	-5.2	0.48	-5.0	0.28	0.25	22.00
Inoculated, 1 cc. bacteria, 0.75 cc. invertase.....	22.00	-4.6	-5.7	1.28	-4.7	0.44	1.06	21.17
Inoculated, 0.5 cc. invertase.....	22.08	-3.1	-5.5	2.30	-4.1	1.09	1.53	20.00
0.25 cc. invertase.....	22.08	-2.0	-5.6	3.24	-2.5	1.40	2.28	16.40
0.1 cc. invertase.....	23.19	-2.5	-5.5	3.91	-3.5	1.04	2.18	16.40
Control.....	22.14	21.0	-5.7	21.27	-5.4	20.75		

sponds to 0.787 of the specific rotation of sucrose, which is 66.5. Hence 1.27 per cent of gum will indicate on hydrolysis by the Clerget method of inversion 1 per cent of sucrose in excess of the amount actually present. In the following tables are given the results of the experiments on the production of gum levan by bacteria in the presence of invertase. The abbreviations used in the tables are as follows:

S.P. = single polarization
 I.R. = invert reading
 S.C. = sucrose clerget
 R.S. = per cent reducing sugars

The results in table 1 show that the production of gum by the bacteria is in inverse proportion to the amount of invertase added. In only one case did this inverse relationship between invertase added, and gum produced, fail to exist, and that was in the case of the addition of 0.25 and 0.1 cc. These results agree fairly well with those we obtained in our earlier work (1911 a, 1918) in which we used joint inoculations of yeast and bacteria. It is obvious that in both cases the transformation of sucrose, either by invertase added direct, or secreted by the yeast, tends to deprive the bacteria of the material from which they produce gum levan. The following table shows the results

TABLE 2

Results of experiment in which gum production in the presence of yeast and bacteria was determined

SERIES	CULTURE	ACIDITY— 20 CC. USED; CC. N/10 K.OH REQUIRED	REDUC- ING SUGAR	SINGLE POLARI- ZATION	SUCROSE (CLERGET)	GUM
	Control.....	0.7		9.3	9.73	
A. (1)	B. IV.....	4.1	4.25	4.5	5.89	2.69
B. (1)	B. IV and yeast.....	8.5	7.02	-0.8	2.08	1.47
C. (1)	Yeast.....	6.6	7.28	-0.6	2.32	
	Control.....	0.8		9.3	9.44	
A. (2)	B. IV.....	3.8	6.78	-0.3	1.88	4.85
B. (2)	B. IV and yeast.....	9.1	2.27	-2.8		0.39
C. (2)	Yeast.....	5.4	6.25	-4.5		

of one of the earlier experiments in which gum production in the presence of yeast and bacteria was determined.

In table 2 it will be seen that the presence of the yeast in the sucrose solution decreased the amount of gum produced by the bacteria by approximately 50 per cent in one case and almost 90 per cent in another.

In studying the production of gum levan by bacteria in the presence of invertase, it is of great importance to so regulate the amount of invertase added that the inversion will proceed at a regular rate throughout the entire incubation period. If the process is too rapid and glucose and levulose are formed faster than the bacteria can utilize them while in the nascent con-

dition, then the addition of the invertase might be regarded as a disadvantage to the bacteria, even in the light of Greig Smith's theory. In the following experiment the rate of action of the varying amounts of invertase added was measured, and the results are given in table 3.

It will be noted from the results given in table 3 that while the addition of 1 cc. of invertase resulted in an inversion of

TABLE 3

The results of daily analyses showing the rate of action of invertase in experiment 2

SAMPLE	FIRST 24 HOURS		48 HOURS		72 HOURS		96 HOURS		120 HOURS		168 HOURS	
	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.
1 cc. invertase....	14.7	15.87	11.5	13.37	8.3	10.99	6.0	8.5	4.2	7.92	0.3	4.72
0.5 cc. invertase..	17.1	17.82	15.3	16.27	9.7	12.05						
0.25 cc. invertase.	18.3	18.75	17.3	18.13	14.8	16.1	12.6	14.42	10.8	13.07	6.4	8.5
0.1 cc. invertase..	19.0	19.38	18.2	18.65	17.4	18.04	16.1	17.20	15.0	16.3	12.6	14.46

TABLE 4

The results of experiment 2 on the formation of gum levan in the presence of invertase

SAMPLE	TOTAL SOLIDS BY REFRACTO- METER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
No invertase, 1 cc. bac- teria.....	21.9	-8.6	2.9	11.92	-2.1	9.37	3.23	8
0.1 cc. invertase.....	21.9	-3.4	-3.3	2.92	-2.5	1.60	1.67	18
0.25 cc. invertase.....	21.9	-4.0	-3.0	1.96	-2.3	0.7	1.60	17
0.5 cc. invertase.....	21.9	-6.0	-3.2	0.7	-3.1	0.62	0.1	20
1 cc. invertase.....	21.9	6.6	-3.0	0	-3.2			20
Control.....	21.3	19.0	-5.8	19.27	-3.3	20		

sucrose at the rate of 2.5 per cent per day, the addition of 0.5 per cent caused inversion at the rate of 1.5 per cent of sucrose daily, and the smaller additions at correspondingly slower rates. While the addition of 1 cc. would have resulted in the exhaustion of the sucrose before the end of the ten-day incubation period, the smaller additions should have furnished ideal conditions for the maintenance of a constant supply of nascent glucose and levulose. The results of the experiment are given in table 4.

The results in table 4 tend still further to confirm the theory that sucrose, rather than the nascent products of its inversion, is the source of gum levan. If we compare the rate of action of the various additions of invertase as shown in the above table, with those shown in the previous one, where the invertase was acting in the absence of bacteria, we find that the rate of action was much more rapid in the present experiment. For example, where 1 cc. of invertase had been used alone, only about 16 per cent of the sucrose was inverted in one week, while the same amount when used with the bacterial inoculation resulted in the almost complete inversion of the sucrose in a period of ten days. The more rapid action under the latter conditions is due to the production of acids by the bacteria, which makes the condition more favorable for invertase action. The rate of action of invertase in the presence and absence of bacteria was compared in the next experiment, in which larger inoculations of bacteria were used. Instead of a platinum loop, 1 cc. portions of a 48-hour old bouillon culture were employed, in order that the bacteria might have a better opportunity to utilize the invert sugar as rapidly as it is formed. The results are given in table 5.

The accelerative action of the growing bacteria upon the inversion of sucrose by the added invertase is very clearly shown in table 5. While 0.25 cc. of invertase had only inverted about 16 per cent of the sucrose in the 20 per cent solution in one week, the addition of an equal quantity of invertase had inverted 85 per cent of the sucrose when acting in the presence of bacteria for the same length of time. While larger inoculations of bacteria had resulted in an increase in gum production as compared with previous experiments, the ratio between the amounts formed in the presence and absence of invertase was similar to that found in the previous experiments.

The additions of the small amounts of invertase used in the previous experiments could scarcely be expected to inhibit the development of bacteria. However, this possibility was tested in the following experiment, in which the solutions were plated out on a 10 per cent sucrose agar at the end of the incubation period.

TABLE 5
The results of experiment 3 showing the rate of action of invertase in the inoculated and uninoculated sucrose solutions

SAMPLE	48 HOURS			72 HOURS			96 HOURS			144 HOURS			7 DAYS		
	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.
A. invertase only	17.3	-6.3	18.4	15.9	-6.4	17.42	13.8	-5.5	15.20	9.9	-6.2	12.89	9.0	-6.7	12.38
B. 0.25 cc. invertase, 1 cc. bacteria....	16.4	-6.3	17.69	12.3	-6.3	14.59	6.2	-5.9	9.71	-2.4	-6.1	3.31	-3.0	-6.3	2.97
C. bacteria only	20.0	-6.4	20.5	19.3	-6.2	19.7	17.7	-5.7	18.3	9.4	-5.8	11.23	6.0	-6.3	9.78
	8 DAYS			10 DAYS			PER CENT R.S.	INVERTASE			PERCENT GUM	SOLIDS			
	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.		S.P.	I.R.	S.C.					
A. invertase only	7.0	-6.5	10.74	5.4	-3.5	9.96	12.9					23.02			
B. 0.25 cc. invertase, 1 cc. bacteria....	3.6	-6.4	2.61	-4.6	-3.3	2.02	20.0				2.33	23.05			
C. bacteria only	3.4	-6.6	8.01	-8.0	-3.4	5.11	14.9				5.54	22.75			

It will be observed from table 6 that the numbers of bacteria in the solutions containing invertase are higher in some cases than where no invertase is present. Since the largest number of bacteria occurred in one case where the largest amount of invertase had been added, the apparent decreases in the other cases where smaller amounts of invertase had been added, cannot be attributed to the inhibiting action of the invertase. Even in the flasks where the bacteria occurred in the smallest numbers,

TABLE 6

The results of experiment 4 showing the development of bacteria and the production of gum levan in the presence and absence of invertase

SAMPLE	TOTAL SOLIDS BY RE-FRACTOMETER	NUMBER PER CUBIC CENTIMETER	S.P.	I.R.	S.C.	PERCENT R.S.	I.R.	S.C.	PER CENT GUM
Control.....	23.12		20.8	-6.1	21.15	Trace	-5.5	20.77	
Inoculated, 1 cc. bacteria only.....	22.92	11,800,000	-0.8	-6.0	4.42	15.3	-2.2	1.24	4.03
Inoculated, + 1 cc. invertase.....	23.12	14,200,000	-2.6	-6.3	3.3	16.9	-1.9	1.21	2.65
Inoculated, + 0.25 cc. invertase.....	23.16	9,320,000	-4.9	-6.3	1.54	20.0	-5.0	0.57	1.27
Inoculated, + 0.5 cc. invertase.....	23.46	11,400,000	-5.8	-6.3	1.06	20.4	-5.5	0.1	1.21
Inoculated, 1 cc. invertase.....	23.49	7,710,000	-6.5	-6.4	0.41	20.6	-5.5	0	0

they were sufficiently numerous to have produced large amounts of gum levan, had other conditions been favorable.

The larger inoculations of bacteria used in this experiment resulted in the production of gum levan in the solution containing 0.5 cc. of invertase, whereas in a previous experiment only a trace of gum had been formed by the bacteria in the presence of that amount of the enzyme.

The results of the previous experiments show that not only does invertase decrease the amount of gum formed, in proportion to the amount of sucrose inverted, but that the remaining

sucrose is utilized to a smaller extent than would be the case if this same amount of sucrose had been available to the bacteria, with no invertase present. This would indicate that the products of the inversion of sucrose hinder rather than aid the bacteria in the production of gum. This fact was observed in our earlier investigations in which various mixtures of sucrose and glucose and levulose were used. The results from those experiments showed that gum production was decreased by the presence of invert sugars, or in other words, that a high ratio between sucrose and invert sugar is most favorable for this type of ferment-

TABLE 7

The results of experiment 5 showing the production of gum levan in the presence of reducing sugars

SAMPLE	TOTAL SOLIDS BY RE-FRACTOMETER	S.P.	I.R.	S.C.	PER CENT R.S.	INVERTASE		PER CENT GUM
						I.R.	S.C.	
Inoculated bacteria	22.53	7.8	-6.0	10.97	10.8	-2.86	8.5	3.13
0.25 cc. invertase, inoculated, bacteria	22.82	-5.3	-6.2	1.15	19.5	-4.18		1.55
Half inverted solution, inoculated, bacteria	17.19	+3.2	-4.7	6.38	10.0	-5.06	6.3	
Control sucrose solution	21.70	18.8	-6.0	19.29	Trace	-6.6	19.24	
Control, half inverted solution...	17.25	8.4	-4.6	10.26	5.7	-5.2	10.50	
Half inverted solution, inoculated, bacteria 0.25 cc. invertase	17.22	-4.8	-4.7	0.28	16.4	-4.62	0	

tation. In order to test this point further, the following experiment was conducted. A 20 per cent sucrose solution was inverted by the addition of 4.5 cc. of invertase to 850 cc. of the solution, and allowing it to stand in an incubator at 32°C. for two days. At the end of this period its sucrose content was 2.24, and it was then mixed with an equal volume of an uninverted 20 per cent Greig Smith solution, containing all of the nutrient material in such proportions that after mixing the two solutions, the mixture would conform to the original formula. This solution was then flaked and inoculated, and compared with

a 20 per cent sucrose solution inoculated in a similar manner, and kept under the same conditions.

It will be seen from table 7 that the bacteria formed no gum in the solution containing invert sugar and sucrose, although there was 10 per cent of sucrose present. The relative amounts of gum formed in the sucrose solution with and without invertase were approximately the same as in the previous experiments.

THE PRODUCTION OF GUM LEVAN UNDER VARYING CONDITIONS OF H-ION CONCENTRATION

In the preceding experiments the solution used was approximately neutral in reaction. In previous investigations it had been found that a neutral reaction was most favorable for the gum levan fermentation, but the optimum H-ion concentration had not been determined. In order to determine this point, and to learn whether the optimum for gum production and invertase action coincided, the following experiment was conducted. A 10 per cent sucrose solution (Smith) was prepared and flasks in 200 cc. portions. These were sterilized by the intermittent method, and after the third sterilization and while the contents of the flasks were still warm, measured portions of N/1 NaOH and H_2SO_4 were added. The flasks were then inoculated the invertase added as before. At the end of the ten-day period and analyses were made and the pH values determined on the controls by the potentiometer. The results are given in table 8.

The pH range in this series (table 8) varied from 6.7 to 9.5, and the greatest gum production occurred in the more acid series, when invertase was absent, but in the presence of invertase the amount of gum produced in that series was less than one-tenth of that produced in its absence. In the series containing 0.5 cc. of N/1 NaOH, the gum produced in the presence of invertase was approximately half as great as that produced in the absence of invertase, and as the NaOH was increased a point was reached where the invertase was inactive, and the gum produced in its presence actually greater than where no invertase was added. This beneficial effect of the invertase was probably due to the fact that it protected the bacteria against the action

TABLE 8

The results of experiment 6 showing the development of gum in the presence and absence of invertase in solutions of varying H-ion concentration

SAMPLE	pH	TOTAL SOLIDS BY RE-FRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
						I.R.	S.C.		
Blank, inoculated <i>B. vulgaris</i>	7.4	11.49	-1.0	-2.8	1.66	-1.6	0.4	1.98	7.75
Blank, inoculated culture XI.	7.4	11.27	-1.2	-2.8	1.00	-1.6	0.28	1.80	8.29
Blank, 0.5 cc. invertase inoculated, culture XI.....	7.4	11.23	-2.8	-3.0	0.38	-2.0		0.48	8.82
0.5 NaOH, inoculated, culture XI.....	8.6	11.27	-1.2	-2.4	1.10	-1.6	0.36	1.34	7.86
0.5 NaOH, 0.5 cc. invertase, Culture XI.....	8.6	11.66	-2.0	-2.4	0.49	-1.6		0.62	8.54
0.5 NaOH, inoculated, <i>vulgaris</i>	8.6	11.60	-1.6	-2.8	1.15	-1.6		1.77	8.77
0.5 NaOH, control, 0.5 cc. invertase.....	8.6	11.66	8.0	-3.6	9.18	-2.4	8.3		2.00
1.2 cc. NaOH, inoculated, culture XI.....	9.5	11.60	1.6	-3.6	1.81	-1.2		2.29	7.24
1.2 cc. NaOH, inoculated, 0.5 cc. culture XI, invertase...	9.5	11.63	-1.6	-3.8	1.98	-1.6		2.51	
1.2 NaOH, inoculated, <i>vulgaris</i>	9.5	11.33	9.2	-4.0	10.44	-2.8	9.65	1.00	
1.2 NaOH, control, 0.5 cc. invertase.....	9.5	11.66	8.8	-3.8	9.97				
2.4 NaOH, inoculated, culture XI.....		11.63	10.0	3.6	10.72	-3.2	10.6		
2.4 NaOH, 0.5 cc. invertase....		11.37	10.0	3.6	10.72	-3.6	10.9		
2.4 NaOH, 0.5 cc. invertase, control.....		11.70	10.0	-3.6	10.72	-2.8	10.2		
4.8 NaOH, inoculated, culture XI.....		11.37	10.0	-4.0	11.06	-2.8	10.2		
4.8 NaOH, 0.5 cc. invertase, control.....		11.70	9.6	-3.6	10.41	-3.2	10.27		
1.2 cc. N/2H ₂ SO ₄ , inoculated, culture XI.....	6.7	11.40	-1.2	-3.6	2.12	-1.2		2.57	9.26
1.2 N/2 H ₂ SO ₄ , inoculated, <i>B. vulgaris</i>	6.7	11.40	-1.6	-3.6	1.81	-1.2		2.29	9.26
1.2 N/2 H ₂ SO ₄ , 0.5 cc. invertase, inoculated, culture XI.....	6.7	11.63	-3.2	3.2	0.41	-1.6		0.17	8.33
1.2 N/2 H ₂ SO ₄ , 0.5 cc. invertase, control.....	6.7	11.66	-2.8	-4.0	1.22	-2.8	0.2	0.129	9.2
Control.....	6.7	11.40	10.4	2.0	11.3	-0.7	10.5		
Control.....	6.7	11.70	10.0	2.6	11.06	-0.7	10.24		

of the alkali. In the next experiment a series of solutions were prepared having a pH range from 4.8 to 9.5.¹ The results are given in table 9.

The maximum gum production in this experiment (table 9) occurred in the series with a pH of 7.4. The gum production in

TABLE 9

The results of experiment 7 showing the development of gum in the presence and absence of invertase in solutions of varying pH

SAMPLE	pH	TOTAL SOLIDS BY RE- FRACTOMETER	S.P.	ACID INVERSION		INVERTASE		PERCENT GUM	PERCENT R.S.
				I.R.	S.C.	I.R.	S.C.		
Blank, inoculated, <i>B. vulgatus</i>	7.4	12.44	-2.8	-7.2	3.90	-4.0	1.2	3.43	9.00
Blank, inoculated, <i>B. vulgatus</i> , 0.5 cc. invertase	7.4	13.09	-4.4	-4.8	0.67	-1.1	0.33	0.42	12.5
Blank, not inoculated, 0.5 cc. invertase	7.4	13.09	-4.0	-7.2	2.99	-6.4	2.35		11.76
Blank, control	7.4	12.44	9.6	-4.8	11.38	-4.0	10.7		
1.2 cc. NaOH, 200 cc., inocu- lated, <i>B. vulgatus</i>	9.5	12.69	6.0	-5.6	9.30	-4.0	8.08	1.54	2.74
1.2 cc. NaOH, inoculated, cul- ture XI, 0.5 cc. invertase . . .	9.5	12.48	-2.4	-5.6	2.87	-4.0	1.5	1.60	7.14
1.2 NaOH, inoculated, culture XI	9.5	12.48	9.6	-6.4	10.07	-4.4	11.1		
0.5 cc. invertase, 1.2 cc. NaOH, not inoculated	9.5	12.48	8.4	-5.6	8.78	4.8	10.5		1.37
2.4 cc. N/2H ₂ SO ₄ , inoculated, culture XI	4.8	12.48	4.4	-4.8	7.40	-4.4	5.25	2.73	3.80
2.4 cc. N/2H ₂ SO ₄ , 0.5 cc. in- vertase inoculated, culture XI	4.8	13.13	-3.2	-4.0	0.91	-4.4	1.21	0.88	12.12
2.4 cc. N/2 H ₂ SO ₄ , not inocu- lated, 0.5 cc. invertase	4.8	12.69	-4.4	-4.0	0	-4.4	0.3		11.76

the series with a pH of 4.8 was greater than in the series having a pH of 9.5. These results show that while the bacteria forming

¹ The writer is indebted to Dr. J. F. Brewster and Mr. W. G. Raines, Jr., of the research Chemical Department, for their kind coöperation in making the pH determinations in the above experiments.

levan from sucrose have a wide range for their activities, their optimum pH does not coincide with the optimum for invertase action. In the presence of invertase, the maximum gum production occurs under conditions where the pH most completely restrains inversion, and the minimum gum production occurs where the conditions are most favorable for it.

THE PRODUCTION OF GUM LEVAN IN RAW AND CLARIFIED SUGAR CANE JUICE

The gum fermentation of cane juices is frequently a problem of considerable economic importance, since it renders the product much more difficult to clarify, and decreases the yields of sugar obtainable from it. An experiment was carried out to determine the relative susceptibility of raw, sulphured, and limed juice to this type of fermentation. A large supply of raw juice was obtained, and divided into two parts, one of which was sulphured to 5.9 cc. (10 cc. of the juice requiring 5.9 cc. of N/10 NaOH to neutralize it). A portion of this sulphured juice was then treated with milk of lime until the acidity had been reduced to 0.5 cc. The original untreated juice made up the third series of the experiment. The pH range was from 3.8 to 6.9. The samples were sterilized on three successive days at 30°, inoculated and incubated as in previous experiments.

The results of table 10 show that the production of gum levan by all three of the cultures used was greater in the limed than in either the raw or the sulphured juice. While the pH of the raw juice was not determined, the average for Louisiana juices is about 5. The acidity of this sample was 1.85 cc., which would indicate a normal juice. From these data it is clear that a clarified juice is much more favorable in its reaction for the production of gum levan than either raw or sulphured juices.

THE SPECIES OF BACTERIA CONCERNED IN THE PRODUCTION OF LEVAN FROM SUCROSE

We have already referred to the fact that Greig Smith (1901, 1902 a), who was one of the first to investigate the bacterial

flora of sugars, gave the name of *Bacillus levaniformans* to the gum forming bacteria that he isolated therefrom. He pointed out the marked similarities between this species and the potato bacillus, especially in regard to the great resistance of its spores to heat, and the characteristic mesentery like growth upon potatoes. The writer in his previous investigation of this sub-

TABLE 10

The results of experiment 7, showing the formation of gum levan in raw, sulphured, and limed cane juice

SAMPLE	TOTAL SOLIDS BY RE-FRACTOMETER	PER CENT R. S.	ACID INVERSION SUCROSE			INVERTASE SUCROSE		PER CENT GUM
			S.P.	I.R.	S.C.	I.R.	S.C.	
1.85 cc. Acid, raw juice, control.....	16.50	4.00	9.82	4.20	11.09	4.4	11.3	
Raw juice, inoculated, culture XI	15.64	10.4	-0.2	-2.8	2.21	-1.2	1.8	0.6
Raw juice, inoculated, <i>B. vulgatus</i>	14.81	10.0	-0.2	-0.4	0.52	-0.6	0.86	
Raw juice, inoculated, potato culture.....	14.51	7.5	1.4	-4.4	2.65	-1.6	1.6	1.33
Sulphured to 1 cc., 5.9 cc. acid, control.....	16.96	14.28	2.2	1.61	1.01	1.8	1.2	
Sulphured, inoculated, potato culture.....	16.96	13.7	2.00	1.80	1.51	2.0	1.7	
Sulphured, inoculated, <i>B. vulgatus</i>	16.96	13.2	2.2	2.05	1.85	2.0	1.7	
Sulphured, inoculated, culture XI.....	16.70	12.7	2.2	1.60	1.01	2.0	1.7	
Limed, 0.5 cc. acid, cold, control.	16.70	3.14	10.6	-1.8	11.18	-1.6	11.40	
Limed, inoculated, <i>B. vulgatus</i> ..	16.70	11.4	2.6	2.2	5.72	-1.6	3.37	2.98
Limed, inoculated potato culture	14.85	7.28	1.4	4.8	5.14	-1.6	2.4	3.47
Inoculated, culture XI.....	16.70	10.6	2.2	-2.2	5.41	-3.2	4.42	1.28

ject found that *Bacillus vulgatus* obtained from the Museum of Natural History of New York City, had the ability to produce gum levan from sucrose, and was in all other respects identical with the cultures isolated from sugars. The other species belonging to the potato group of bacteria, viz., *Bacillus mesentericus-fuscus* and *Bacillus liodermos*, seemed to have little if any such ability. It was found that this levan-forming ability on the

part of *Bacillus vulgatus* could be rapidly increased by frequent transfers in sucrose solutions. After once acquiring this ability, the microorganism retains it apparently, with but little loss. As the gum determinations in the previous investigation were made simply by a measure of the viscosity of the solution, it was decided to repeat them in connection with this investigation, using a more reliable method of gum determination. For this purpose cultures of *Bacillus vulgatus-mesentericus fuscus* and *B. liodermos* were obtained from the Museum of Natural History New York, through the courtesy of Dr. C.-E. A. Winslow. These cultures were used to inoculate flasks containing 200 cc. of a 10 per cent sucrose solution. The inoculations were made from tubes of the same solution, transfers from which were made to fresh sterile solution every forty-eight hours. The cultures that were obtained from sugar were transferred similarly to tubes of sterile bouillon containing no sucrose, or other sugar. These transfers were kept up for a week, at the end of which time a second set of flasks were inoculated. Table 11 shows the production of gum in the first experiment.

The results in table 11 show that the production of gum levan by *Bacillus vulgatus* is much less than that produced by the cultures obtained from sugars. An old culture that had been kept in the form of a spore preparation on a cover glass, produced almost twice as much gum as the former. The latter culture had been repeatedly used in sucrose solutions.

After transplanting the culture as above described, a second inoculation was made with the results indicated in table 12.

From the results of this experiment (table 12), it will be seen that the production of gum by *Bacillus vulgatus* is exactly three times as great as in the former case. There was considerable variation in the effect of the repeated propagation upon the gum-forming powers of the cultures from sugar. As the first experiment might not have been entirely representative, a comparison with the following experiment (table 13) will probably offer a more reliable indication of the effect of these transplantations in a substratum containing no sugars.

TABLE 11

The results of experiment 8, showing the production of gum from sucrose by *Bacillus vulgatus* when first grown in sucrose solution

CULTURE	TOTAL SOLIDS BY RE-FRACTOMETER	S.P.	I.R.	S.C.	PERCENT R.S.	INVERTASE		PERCENT GUM
						I.R.	S.C.	
VII.....	13.16	-3.0	-2.15	1.36	5.4	-1.5	0.2	1.39
XI.....	12.17	-2.8	-1.85	0.95	7.6	-1.4	0.2	0.88
XVI.....	13.19	-2.2	-2.0	1.65	7.09	-1.1	0.01	2.08
XVII.....	13.94	-1.6	-2.4	2.76	7.4	-1.2	0.07	3.41
Old culture, <i>B. vulgatus</i>	13.19	-3.4	-2.2	1.08	9.0	-1.2	0	1.37
Control.....	13.84	8.6	-2.6	10.83		-3.3	11.5	
<i>B. vulgatus</i>	13.74	7.6	-2.4	9.70	0.9	-2.0	9.1	0.7
Culture I, old series, spore preparation.....	14.09	1.0	-2.4	4.73	6.9	-1.5	1.7	3.84
Culture II.....	13.74	-3.0	-2.2	1.38	8.7	-1.5	0.23	1.46
Culture IV.....	13.51	-0.8	-2.35	3.3	8.6	-1.7	2.2	1.39
Culture V.....	14.16	-3.4	-2.2	1.08	8.7	-1.6	0.09	1.25
Culture VI.....	15.70	-4.0	-2.6	1.29	8.54	-1.6	0	1.63

TABLE 12

The results of experiment 9, showing the increased ability to produce gum levan by *Bacillus vulgatus* after repeated transfers in sucrose solutions

SAMPLE CULTURE	TOTAL SOLIDS BY RE-FRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PERCENT GUM	PERCENT R.S.
					I.R.	S.C.		
Culture I.....	11.69	+0.6	-4.0	3.8	-1.4	1.65	2.73	7.5
Culture II.....	10.36	-1.2	-3.2	1.78	-1.2		2.27	7.27
Culture IV.....	11.60	-1.8	-2.8	0.9	-2.4	0.6		9.0
Culture V.....	10.36	0.8	-4.8	4.58	-2.8	3.00	2.00	5.8
Culture VI.....	11.67	-1.2	-4.8	3.04	-2.8	1.45	1.91	7.5
Culture XI.....	11.08	-0.4	-4.8	3.74	-2.2	1.39	2.98	8.7
Culture XIII.....	11.45	2.6	-4.4	5.69	-2.8	4.3	1.86	5.9
Culture XIV.....	11.45	-1.0	-5.4	3.78	-2.0	0.9	3.75	8.5
Culture XVI.....	11.45	-2.4	-3.6	1.19	-2.0	0	1.51	8.7
<i>Vulgatus</i>	11.01	2.0	-4.8	5.57	-2.8	3.91	2.10	3.5
<i>Mss. fuscus</i>	10.50	7.8	-3.2	8.66	-3.4	8.8		
Old <i>vulgatus</i>	10.43	-1.6	-4.4	2.48	-2.8	1.18	1.65	7.4
<i>Liodermos</i>	10.22	8.6	-3.0	9.14	1.79	5.5		

It is particularly interesting to note from the results in table 13 that six of the cultures isolated from sugars had decreased in their levan-forming power, and one seemed to have lost it entirely. The old *Bacillus vulgatus* produced only one-fourth as much gum as in the first experiment, and no further increase resulted from the transfers of the new *Bacillus vulgatus* culture.

TABLE 13

The results of experiment 10, showing the effect of repeated transfers in sucrose and nonsucrose media upon the ability of Bacillus vulgatus to produce gum levan

SAMPLE	TOTAL SOLIDS BY RE-FRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
Culture I, no phosphate	12.85	2.4	-2.8	4.09	-0.8	2.5	2.01	8.5
Culture II, phosphate media	12.95	3.2	-4.0	5.72	1.2	3.48	2.84	9.09
Culture II, phosphate	13.22	1.6	-5.2	5.51	-2.0	2.94	3.26	8.11
Culture II, no phosphate	13.23	2.4	0.9	4.79	-2.0	3.56	1.56	7.33
Culture IV	13.23	1.6	1.1	4.85	-2.8	3.62	1.56	8.69
Culture V	13.23	2.4	-1.2	5.78	-2.8	4.24	1.95	7.84
Culture XI	13.23	1.6	1.1	4.85	-1.2	2.26	3.30	8.69
Culture XIII	13.23	11.2	0.9	11.4	-3.2	11.39		
Culture XIV	12.27	1.6	1.1	5.78	-2.4	3.28	3.17	9.09
Culture XVI	11.66	10.4	1.1	10.53	-2.8	10.39		
Old culture, vulgatus	11.66	2.4	0.9	4.81	-3.2	4.56	0.32	6.94
Culture, <i>B. vulgatus</i>	12.31	3.2	1.1	5.32	-1.6	3.82	1.90	7.89
<i>B. liodermos</i>	11.66	11.2	1.1	11.8	-3.2	11.3		
<i>B. mesentericus</i>	11.36	11.2	1.0	11.8	-3.6	11.6		
Culture from potato, 2 transfers	12.56	3.2	1.1	6.08	-2.8	4.84	1.44	7.24
Control	11.67	2.6	1.3	10.49	-3.2	10.6		

From these results one is led to conclude that the production of gum levan by the *Bacillus vulgatus* is a power that is easily acquired and fairly easily lost, until it has become thoroughly established, when it appears that it is held rather tenaciously. This would explain why the old culture of *Bacillus vulgatus* almost entirely lost its gum-producing power, when grown in sugar free media, and why some of the cultures from sugar reacted in a similar way, and why others retained their power

under these same conditions. In addition to being a test of acquiring and losing gum-producing power, this experiment is also a test of the value of phosphates in promoting this type of fermentation. The first four lines of the table show a comparison of gum formation by culture no. 1 and 11 in a solution with and without phosphates. It will be observed that the presence of phosphates greatly promotes gum formation. In the latter part of the table it will be noted that a culture freshly isolated from potato was used, and that it produced 1.44 per cent of gum. The method used to isolate the culture was as follows: A potato was thoroughly cleaned on the outside with a brush. The peelings were then put in a 300 cc. Erlenmeyer flask containing some sterile sucrose solution. The flask was then placed in the incubator, and after growth had taken place, transfers were made to tubes of the same medium. Sucrose agar plates were then made and a pure culture obtained.

It will be noted that neither in this nor in the previous experiment was any gum formed by *Bacillus liodermos* or *Bacillus mesentericus-fuscus*. This is approximately the same conclusion that we reached in the previous investigation to which we have referred.

In order to determine whether the acquisition of gum-producing power is more rapid when the bacteria are transferred to sucrose solutions of higher densities than the 10 per cent that we had been using, a comparison was made between a 10 per cent and a 20 per cent solution.

The cultures used in this experiment (table 14) were the *Bacillus vulgatus* and the culture isolated from a potato, which we had used in the preceding experiment. It will be noted that the gum-producing power of the former culture was much greater after being grown in the 20 per cent sucrose solution. The other culture showed a slightly greater gum-forming power after being grown upon the 10 per cent solution. The results of our earlier investigation had very strongly indicated that the gum-forming power is acquired more rapidly by these species of bacteria, when they are grown in concentrated sucrose solutions.

The conditions for acquiring this property are to be found in cane sugar factories. The gum-forming bacteria are brought to the mill in the trash and particles of soil adhering to the cane. When the cane is crushed they are carried into the juice, and their spores survive the temperatures attained during the boiling and evaporating of the juice, and the crystallization of the sirup.

TABLE 14

The results of experiment 11, showing the relative rate at which gum-forming ability is acquired in 10 and 20 per cent sucrose solutions

SAMPLE	TOTAL SOLIDS BY RE- FRACTOMETER	SP.	I.R.	S.C.	PER CENT GUM	INVERTASE		PER CENT R.S.
						I.R.	S.C.	
Control	12.16	11.0	-3.2	11.02		-2.0	10.43	
Control	11.82	10.8	-2.8	10.54		-2.0	9.9	
Inoculated, <i>B. vulgatus</i> , 10 per cent sucrose solution	11.50	-0.8	-2.4	1.38	1.25	-1.2	0.39	7.35
Inoculated, <i>B. vulgatus</i> , 20 per cent sucrose solution	11.82	-0.8	-4.0	2.70	2.92	-1.2	0.4	7.8
Potato culture, 10 per cent sucrose solution	11.83	-0.4	-5.2	3.99	4.35	-1.2	0.4	7.1
Potato culture, 20 per cent sucrose solution	11.58	+0.4	-5.0	4.4	3.49	-1.6	1.65	6.94
<i>B. mesentericus</i> , 10 per cent sucrose solution	11.07	10.2	-2.6	9.91		-3.6	10.7	
<i>B. mesentericus</i> , 20 per cent sucrose solution	11.58	10.8	-2.9	10.62		-3.2	10.92	
<i>B. liodermos</i> , 10 per cent sucrose solution	11.58	9.8	-2.6	9.61		-2.8	9.8	
<i>B. liodermos</i> , 20 per cent sucrose solution	11.58	10.6	-2.6	10.22		-2.8	10.4	

The great resistance of the spores of these bacteria to heat, and their ability to withstand the osmotic pressure of the high density sirups and molasses to which they are subjected, fit them admirably for this environment. The potato group of bacteria are enabled to withstand this osmotic pressure, owing to the fact that they have permeable (Fischer, 1903) cell membranes, and hence their cell walls are not easily plasmolyzed. It is on this

account that we find some of these species capable of producing gum levan in sucrose solutions of 50 to 60 per cent. We conclude therefore that the soils surrounding cane sugar factories will gradually become inoculated with potato bacteria which have acquired the ability of forming gum levan from sucrose, owing to the practice of returning the filter press cake to the soil. The final molasses from cane factories, when fed to the stock on the plantation, would probably result in infecting the manure with these gum-forming species, which would again find their way back to the soil in the manure. Hence there will be a continuous cycle of acclimatization and distribution of these organisms on a sugar plantation.

THE PRODUCTION OF GUM LEVAN FROM SUCROSE IN ITS RELATION TO THE POTATO GROUP OF BACTERIA

We have already referred to the fact that in morphological and physiological characteristics the bacteria isolated from sugars are, with the exception of the ability to form gum levan from sucrose, identical with the potato species. The potato group of bacteria comprise the following species: *Bacillus vulgatus* (Trevisan), (*Bacillus mesentericus vulgatus* (Flügge) and potato bacillus of various authors), *Bacillus mesentericus fuscus* (Flügge), (*Bacillus mesentericus* (Trevisan) and *Bacillus liodermos* (Flügge), (Chester, 1901)). No data are given by Chester on the behavior of these species in sucrose solutions, although a closely related species *Bacterium panis* (*Bacterium mesentericus, panis-viscosus*), which was first isolated from stringy dough is said to produce a viscous fermentation of bread dough. Migula (1897) classes *Bacillus liodermos* as identical with the gum bacillus of Loeffler. Chester (1903) who studied *Bacillus mesentericus*, mentions only the production of acid in sucrose solutions. However, he also describes a culture of *Bacillus mesentericus fuscus* obtained from the Bacteriological Laboratory of the Johns Hopkins University, which he named *Bacillus subtilis* var. *viscosus*. The following description of the growth of this species on agar very strikingly suggests the species we have under investigation. "Agar cultures have the same thick glassy viscid appearance and the growth

can be drawn out into long threads, or behaves under a needle-like thick mucus." Lohnis (1913) refers to the activities of certain gum-forming varieties of the potato bacillus, in the viscous fermentation of the diffusion juice in beet sugar factories. From a review of the literature on the physiological characteristics of the potato group of bacteria it is apparent that but little attention has been paid to their ability to induce the gum fermentation in sucrose solutions. In view of this fact it can be readily understood how easy it would be to overlook this characteristic of these species, particularly since it seems to be largely an acquired ability. Under these circumstances it is very probable that many of the gum-forming species of bacteria have been given specific names, when they are really only derived types of the potato group of bacteria, which have been developing for successive generations upon sucrose solutions.

CONCERNING THE NATURE OF THE TRANSFORMATION OF SUCROSE INTO GUM LEVAN

The results of this investigation have shown that sucrose is the substance from which gum levan is formed, by the bacteria that we have been studying. It has been shown further that sucrose can supply the needs for levan-forming bacteria only when it exists as such in the culture solution. The presence of invertase decreases gum formation in the exact proportion in which it hydrolyzes the sucrose present. A study of the tables shows also that, in the absence of invertase, the amount of gum formed is always in proportion to the sucrose that has been utilized by the bacteria in the solution. In other words, we have here a true fermentation in which definite products are formed, and which is not to be regarded as the result of an abnormal condition of the bacterial cells. We have already referred to the fact that the species of bacteria concerned in the gum levan fermentation do not have a demonstrable capsule. This indicates that the gum is formed extracellularly. Evidence of the existence of an extracellular gum-forming enzyme in connection with this type has been presented by Beijerinck (1910), who succeeded in producing gum levan extracellularly by

the use of extracts prepared from these same species. The gum fermentation of sucrose is, in the light of our present knowledge, of a two-fold advantage to the species of bacteria inducing it; enabling them in the first place to transform sucrose into an assimilable form, and in the second place reducing the osmotic pressure of the sucrose solution, rendering the environment more favorable for their continued activities. For these purposes this fermentation is of greater advantage to the microorganism than the secretion of invertase would be, because the inversion of the sucrose would offer no protection to the bacteria from the osmotic action of the solution, while its conversion into gum would reduce it in proportion to the amount of this colloid formed. It is not easy to explain how this transformation of the disaccharide sucrose into the polysaccharide gum takes place. Browne (1912) has suggested the possibility of the action being a combined assimilative and enzymic action, the sucrose being assimilated into a higher saccharide, which is broken up by an enzyme into glucose and levan.

CONCLUSIONS

The formation of gum levan from sucrose does not depend upon the action of invertase, as implied in Greig Smith's theory of the nascent glucose and levulose origin of the gum, but on the contrary is entirely prevented by the rapid inversion of sucrose by this enzyme.

The action of invertase not only decreases the production of gum levan to the extent to which it inverts sucrose, but retards the production of this gum from the sucrose remaining in the solution.

Decreasing the ratio of sucrose to invert sugars decreases the production of gum levan.

The optimum pH for the development of gum levan is between 6.7 and 7. The relative differences in the amounts of gum produced by the bacteria in the presence and absence of added invertase, was greatest in the solutions whose pH values were nearest the optimum for the action of invertase, and least where the pH values were least favorable for invertase action.

A culture of *Bacillus vulgaris* was found to have the ability to form gum levan from sucrose, and by repeatedly growing it in the presence of a suitable sucrose solution this ability was greatly increased. It is concluded from our study of the species of levan-forming bacteria occurring in sugars, that they are all derived types from the potato group of bacteria. Many of the similar species described in the literature as distinct species are thought also to be derived types.

The gum fermentation of sucrose is believed to be a distinct type of fermentation, probably acquired as a means by which an organism secreting no invertase may convert the unassimilable disaccharide sucrose into assimilable forms, and into products whose combined osmotic pressure value is lower than that of the original sucrose. In this manner the material for supplying the energy for the organism is provided, and the environment, if its osmotic value is too high, is rendered more favorable for the continued growth and development of the organism.

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A READILY CULTIVABLE VIBRIO, FILTERABLE THROUGH BERKEFELD "V" CANDLES, VIBRIO PERCOLANS (NEW SPECIES)

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During the investigation of certain electrocapillary effects of importance in filtration through laboratory filters, (Mudd 1923), it became desirable to have a filterable living organism whose surface electric potential could be studied in connection with its filterability. Search was accordingly begun, at the suggestion of Prof. S. B. Wolbach, and in October, 1921, an undescribed and easily cultivable vibrio was isolated in the filtrate from hay infusion made from fresh water from near Boston. This vibrio passes readily through Berkefeld "V" candles impervious to *Erythrobacillus prodigiosus* and *V. comma*.

The point of zero potential difference between this vibrio and its medium unfortunately corresponds to a concentration of hydrogen ions so high as to be incompatible with the organism's normal activity, so that it has proved unsuitable for determining the effect on filterability of reversing this interfacial potential. On the other hand, the new vibrio has proved so useful in a variety of ways for testing other factors in filtration that its description seems merited.

This organism is a short, actively motile, comma-shaped to straight, rod with rounded ends, occurring singly or in short chains. There is typically one polar flagellum, though frequently two or three are present. The flagella are rarely bipolar; in length they range from 3 to 7 μ . With the fuchsin encres stain of Nicolle and Morax they are readily demonstrated.

In length this vibrio ranges from 0.5 to 2.5 μ , the large majority being 1.5 to 1.8 μ long, as determined by measurement of photomicrographs and by the use of a filar micrometer. The thickness is about 0.3 to 0.4 μ .

In young cultures (eighteen to twenty hours) filamentous forms are frequently found, some of which are chains of organisms, but in which, in other instances, subdivision is with difficulty, or not at all, detectable. The filaments are typically long slender spirals; in smears, however, they often appear straight or with only slight curvature.

The organism is Gram-negative. It stains with the ordinary laboratory dyes.

There are no spores, and usually the protoplasm, as seen by dark-field illumination and in stained specimens, appears homogeneous, except for one or more fine granules in an occasional organism. However, when a culture some days old, containing many individuals, which have lost motility and presumably vitality, is examined with dark-field illumination many of the non-motile organisms appear to consist of brightly shimmering, coarse granulations. This same coarse granulation has been occasionally noted, in hay infusion too acid for optimum growth, even in a majority of the motile individuals of the culture. Subcultures on media with proper reaction return to the normal appearance.

Very occasionally, in old (ten to twenty days) acid hay infusion cultures, "involution" forms similar to those of *V. comma* are found; large globoid forms which stain faintly, spoon-shaped and clubbed organisms, and forms which show a banded appearance with carbol-fuchsin. However, the appearance of these markedly atypical "involution" forms is not very common—not nearly so common as in the case of *V. comma*.

Our most satisfactory cultures of this organism have been grown on hay infusion of reaction near neutrality. Under optimum conditions, the medium is diffusely clouded in six to ten hours, and in twenty-four hours a pellicle forms, sometimes tough and whitish, sometimes merely a scum which is faintly iridescent. After two to three days a slimy deposit settles out

at the bottom of the tube. When the tube is swirled, this deposit comes up in the form of a coarsely twisted rope, looking much like an inverted water-spout.

In bouillon and in Witte's pepton growth is similar to that in hay infusion, but rather less abundant.

Litmus milk is unchanged, and no pellicle forms, though growth occurs.

At first the vibrio grew with difficulty on nutrient agar; after one or two generations, however, growth became profuse. The typical twenty-four hour colony is round, convex and slightly flattened on top, 1 to 2 mm. in diameter, with entire margin. The consistency of the colonies is mucoid, and they tend to become confluent. By reflected light they appear bluish-white and glistening; viewed by transmitted light under low magnification, they are yellowish and finely granular. Colonies five to six days old, where there is sufficient room for growth, reach 6 to 7 mm. diameter and show a distinct central boss (pl. 1). The deeper agar colonies are lenticular.

There is no liquefaction of gelatin. The character of the colonies is the same as on agar. In a gelatin stab, while growth is good on and near the surface, it is very faint and scattered along the line of the stab. Growth is not so abundant on Loeffler's blood serum as on either agar or gelatin, and there is no digestion of the medium. On potato the colonies are small, white, and slimy. There is no diastatic action on starch. Nitrates are not reduced, nor is gas formed. No indol is produced.

There is no fermentation of the following carbohydrates: glucose, lactose, sucrose, maltose, mannitol, adonitol, rhamnose and xylose. The most favorable temperature for growth is about 30°C.

The vibrio is actively motile, both in fluid media and in suspensions of young cultures from solid media. Examination of eighteen to twenty-four hour hay infusion cultures shows most of the individuals free-swimming, relatively large and slow-moving; a few small groups of agglutinated organisms are to be seen. In older cultures (several days old) agglutinated masses are much more in evidence and the free-swimming organisms are smaller,

a few even coccoid, and motility appears much swifter and more vigorous. In hay infusion cultures, weeks or months old, motility may be almost absent, although subculture proves viability still present.

Progression with rotation on the long axis is the characteristic mode of motion with the vibrio. A greater or less degree of precession, or "wobbling" of the long axis, is also to be seen in a large number of the organisms. The combination of these movements in the small, swiftly moving forms produces a strong impression of a progressive undulation passing down the organisms, which, however, is apparently illusory, since the long, slow-moving, filamentous forms present in young hay infusion cultures can be seen to retain their shape during motion, and to be without "notable" flexibility. During division, when the daughter individuals are separated by an obvious constriction, their axes may be quite movable with reference to each other.

The direction of motion is reversible. An individual may often be seen to dart forward, then backward, then forward again in a slightly different direction in a way suggestive of the avoiding reaction of paramecium.

A filterable spiral form, *Spirillum parvum*, has also been described by von Esmarch (1902). To this, our organism in some ways appears similar. However, *Sp. parvum* does not grow on either potato or milk. Old agar colonies have a violet tinge. Moreover, it grows very slowly, rendering fluid media turbid only after eight or ten days. Among the larger forms of filterable organisms should be mentioned the spirochaetes, both parasitic and free-living, whose passage through the Berkefeld filter Wolbach (1915) has demonstrated, and the closely allied Leptospirae (Noguchi, 1918, 1919). In view of the distinctive morphological and cultural characteristics of the new organism and its filterability, we propose the name *Vibrio percolans*.

An agglutinating serum for *V. percolans* can be produced in guinea pigs by intraperitoneal injections of 2 cc. of a twenty-four bouillon culture of the organism every other day for fourteen days. This serum, if allowed to act for twenty-four hours at room temperature, agglutinates *V. percolans* in dilutions up to

1:750. Normal guinea-pig serum has no agglutinating action on the organism. *V. percolans* agglutinating serum has no effect on *V. comma*, *Sp. Finkler-Prior*, or *Sp. metchnikoffi*.

Table 1 summarizes the results obtained in determining the thermal death point of *V. percolans*. The cultures used were twenty-four hours old in the bouillon of $(H^+) = 1.3 \times 10^{-7}$ (pH = 6.9). Approximately one and a half minutes was required to raise the culture to the given temperature. The results were read after forty-eight hours incubation of the subcultures at 30°C.

TABLE 1

DEGREES CENTIGRADE	TIME														
	½ minute	1 minute	1½ minutes	2 minutes	2½ minutes	3 minutes	4 minutes	5 minutes	6 minutes	8 minutes	9 minutes	12 minutes	15 minutes	16 minutes	20 minutes
56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
54	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
53	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0
52	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
51	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates that the subcultures made at the time specified gave a growth of the vibrio; 0 indicates negative subculture.

The organism is fairly resistant under various conditions. It survived after at least eight days at 0°C. in bouillon. At laboratory temperature one glucose broth culture, plugged merely with cotton, was viable after two months. One of the original subcultures, kept at room temperature on a paraffin plugged agar slant, is alive after seven months. Subcultures made from hay infusion cultures kept only with cotton plugs at laboratory temperature for more than six months in several instances have been positive, as is illustrated by the following protocols.

Large test tube (3.5 cm. diameter) of hay infusion inoculated December 11, 1921 with *V. percolans*. Kept in laboratory with cotton plug until June 29, 1922. Then had evaporated to a little less than

half original volume. Short, medium and filamentous forms of *V. percolans* to be seen under dark field, a very occasional individual motile. Subculture in twenty-four hours swarming with vibrios of high motility.

Test tube (2.5 cm. diameter) of hay infusion inoculated November 26, 1921. By June 29, 1922, had evaporated to a little less than a quarter original volume; opaque, chocolate color; under dark field, heavy suspension of vibrios, an occasional one still motile. Subculture positive.

Test tube (2 cm. diameter) inoculated October 11, 1921. By June 29, 1922, evaporated to a little less than quarter original volume occasional motile vibrio present. Subculture positive.

This vibrio is not pathogenic for white mice, white rats, wild rats (*Rattus norvegicus*), guinea-pigs, and rabbits, either by subcutaneous or intraperitoneal injection or by ingestion. One of us (S. W.) swallowed 5 cc. of a twenty-hour broth culture without detecting any effect.

We would call attention to the photomicrographs illustrating this paper and the following paper taken by means of the apparatus for ultra-violet photomicrography kindly placed at our disposal by the late Prof. H. C. Ernst.

The light source is the 2800 Å. line of a magnesium arc. By means of an apparatus for mechanical focussing, devised by Dr. W. T. Bovie, it is possible to focus accurately on the bacteria, using for illumination the harmless green portion of the magnesium spectrum, and then to change directly to the proper focus for ultra-violet light. In this way, the organisms are subjected to ultra-violet light during only the actual exposure of the plate (three to five seconds).

In order to be sure the organisms were not killed by this exposure, the photographed preparations, which were made in 0.5 per cent agar in saline, were rubbed up in sterile broth and plated out. In every case a heavy growth was obtained.

Both from the point of view of theory (V. Sabine (1906) and Ernst and Wolbach (1906)) and of practical results, it is believed that the ultra-violet microscope gives more nearly the actual structure of the living bacterial cell than does any other method of direct observation.

POWER OF LOCOMOTION OF *V. PERCOLANS* AS COMPARED TO THAT OF *V. COMMA* AND *E. PRODIGIOSUS*

In correlation with the study of motility as affecting filter-ability described in the paper subsequent, investigation was made of the passage of *V. percolans*, *V. comma* and *E. prodigiosus* through a coarse-pored filter without aid by any pressure head and dependent solely upon their own powers of locomotion and growth. The following modification of the method of Carnot and Garnier (Besson, 1914) has been used:

A U-tube, 14 mm. inside diameter, with arms 20 cm. long, has a plug of glass wool placed in the bottom of one arm. Above this is placed a layer of 60/120 quartz sand (average diameter of grains 0.23 mm.), acid-washed, about 10 cm. in depth. Hay infusion or nutrient bouillon is poured in, filling both arms to a level one centimeter above the sand, the arms plugged with cotton, and the whole autoclaved. When ready to inoculate, 2 to 3 cc. of the medium is removed from above the sand with a sterile pipette, and replaced with an equal amount of a suspension of the organism to be tested. The tubes are then incubated. Appearance of a faint clouding in the uninoculated arm of the tube indicates the end point. By dividing the time in hours from inoculation to clouding of the sand-free arm by the depth in centimeters of the sand layer, the approximate average time required by the organism to pass 1 cm. of the sand is obtained.

In the experiments each organism was placed under its optimum conditions of growth. *E. prodigiosus* and *V. comma* were grown at 37°C., the former in bouillon and the latter in pepton bouillon. *V. percolans* was grown at 30°C. in faintly alkaline hay infusion. The slightly lower temperature of cultivation may have been a serious handicap to *V. percolans* in comparing its rate of passage with that of *V. comma*; if so *V. percolans* was also handicapped in the filtration experiments in which its temperature was again somewhat lower.

V. comma showed consistently greater speed in effecting passage than did *V. percolans*. The optimum rates obtained were as follows:

ORGANISM	TIME FOR PASSAGE 1 CM. OF SAND
<i>V. comma</i> (Rosebank strain)	1 hour 48 minutes
<i>V. percolans</i> :	
Strain V ₁₈	2 hours 24 minutes
Strain V ₁₉	2 hours 29 minutes
Strain V ₂₉	2 hours 30 minutes

*E. prodigiosus*¹ did not pass the sand in the six experiments performed: three experiments were of one week and three of two weeks' duration.

A culture of *V. percolans* has been deposited in the Army Medical Museum, Washington, D. C., whence any worker interested may obtain a culture.

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¹ The *E. prodigiosus* strain used was from the Medical School stock. Transplants on certain media showed limited motility. In bouillon or suspension from plain agar for instance a few organisms were motile. A culture on blood agar was somewhat more motile. A culture on wet potato showed greatest motility. Of the organism in the fluid at the bottom of the potato tube a considerable number, possibly five per cent, were motile. Motility in hay infusion cultures was not with certainty observed. Such individuals as were motile on any media, with the possible exception of the potato fluid, were quite obviously less vigorous and rapid in their movements than the cholera or percolans vibrios.

EXPLANATION OF PLATE

PLATE 1

FIG. 1. LIVING TWENTY-FOUR HOUR HAY INFUSION CULTURE OF *V. PERCOLANS*.

Ultra-violet light, magnesium line of $\lambda 2800$ Ångstrom units. Five seconds exposure. $\times 1120$.

FIG. 2. PLAIN AGAR COLONIES FIVE DAYS OLD

Natural size. Drawn as seen by reflected light

FIG. 3. PLAIN AGAR COLONIES

Transmitted light; low magnification



FIG. 1

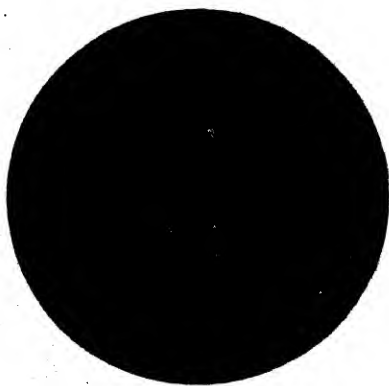


FIG. 2



FIG. 3

FIG. 3

(Mudd and Warren: A readily cultivable vibrio.)

THE PENETRATION OF BACTERIA THROUGH CAPILLARY SPACES

I. MOTILITY AND SIZE AS INFLUENCING FILTERABILITY THROUGH BERKEFELD CANDLES

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The contact surface of the pores of a Berkefeld filter and the water bathing them is the site of an electric potential difference, an ordinary Helmholtz electric double layer, the solid walls taking the negative, and the fluid the positive, charge (Mudd, 1923). If a solution of a "basic" dye, such as methylene blue, is drawn through the filter, electro-positive color ions are taken out by the electro-negative filter capillary walls, and the first portions of the solution may be partially or completely decolorized; more prolonged filtration results in saturation of the filter pores, a colored filtrate and colored wash water. Acid dyes, such as eosine, on the contrary may be filtered without demonstrable change in color; the electronegative color ion does not adhere to the negative capillary walls. The filter is "basophilic."

The filterable microorganism described in the preceding paper, *Vibrio percolans*, in ordinary culture media, was found to be negatively charged with reference to the suspending medium: migration in an electric field, studied with the aid of a microcataphoresis chamber, was toward the anode. The hope was entertained that by acidifying the medium a hydrogen ion concentration could be reached at which the vibrio would still maintain its activity but would carry the positive component of the electric double layer, its filterability thus being suppressed. However vibrios are known to be especially susceptible to acid, (Besson, 1914), and a hydrogen ion concentration of about

5×10^{-6} (pH = 5.3) or above was found to inhibit the motility of *V. percolans*, although insufficiently high to reverse the direction of the surface potential difference. Therefore the question whether or not a filterable microorganism will be found whose filterability can be suppressed by reversal of the interfacial potential with consequent adsorption on the negative filter walls remains an open one.

It was observed, however, that when cultures of *V. percolans* were filtered in media with hydrogen ion concentrations greater than 5×10^{-6} (pH = 5.3) no vibrios grew out in media inoculated with the filtrate; this raised the question whether the motility of a culture could be a determinative factor in its filtration. It has subsequently been found that the passage of *V. percolans* through Berkefeld V candles can be prevented by suppressing its motility with acid, with ether and chloroform, or with cold.¹

SUPPRESSION OF FILTERABILITY OF *V. PERCOLANS* BY ACID

The reaction of the culture of *V. percolans* was at first adjusted by adding a sufficient amount of buffer solution of the desired hydrogen ion concentration. This method in the one experiment tried resulted in stoppage of motility and a negative filtrate; adjustment was made to H-ion concentration of 6×10^{-8} (pH = 4.2). Uncertainty as to possible toxic effect of the buffer ions led to abandonment of this method in favor of a safer one, namely, the washing out of the CO₂ from the culture with a stream of hydrogen and the addition of N/10 HCl until the desired reaction was reached. The hay infusion culture, usually at an initial H-ion concentration not far from 5×10^{-7} (pH = 6.3), by bubbling hydrogen through it for a half to three quarters of an hour could be brought to an approximately stable reaction around 1×10^{-8} , (pH = 8). Acid was then added as desired. The CO₂ could be bubbled from the culture somewhat more quickly if it was kept a little acid by addition of HCl during

¹ By "prevented" is meant prevented at least for intervals slightly more than sufficient to allow the motile control culture to inoculate a filtrate under similar conditions. It is possible, however, that a more prolonged filtration of the culture with motility inhibited might have resulted in inoculation of the filtrate.

the process (Henderson, 1920). The reaction was followed throughout with a Bovie direct-reading potentiometer (Bovie 1915-1916). The condition of the vibrios was followed by observing samples microscopically at frequent intervals with dark field illumination. No change was observed when the cultures were rendered slightly alkaline or acid, but when the H-ion concentration rose above 5×10^{-6} (pH = 5.3) motility became obviously impaired, and above 1×10^{-5} (pH = 5) was practically stopped, save in one culture which had been grown in a medium more acid than usual and in which the H-ion concentration had to be raised to about 4.5×10^{-5} (pH = 4.35) in order to stop locomotion. When motility was observed practically to have ceased the culture was filtered, and, in the seven experiments performed, proved uniformly negative for vibrios; the control filtrates from unacidified cultures were regularly positive, i.e., contained vibrios.

The complete experimental procedure was typically as follows:

The suspension filtered was always a mixed hay infusion culture of *V. percolans* and a weakly motile strain of *Erythrobacillus prodigiosus*. The vibrios were ordinarily the outgrowth in sterile hay infusion tubes of the filtrate from the previous control experiment. The culture was strained if necessary through coarse meshed cloth to remove flocculi and sediment. It was then put in a titration vessel of the Bovie potentiometer, the CO₂ washed out and the hydrogen ion concentration adjusted at from 1 to 5×10^{-6} (pH = 6 to 5.3). Motility of the vibrios at this reaction was still normal as seen by dark field observation. The culture was then divided into two equal parts, the first of which was rendered sufficiently acid to stop locomotion and then filtered, with negative pressure (suction), starting at zero and increasing the pressure difference on the two sides of the filter to several hundred millimeters of mercury; the filtrate was inoculated into sterile hay infusion tubes. Various amounts of the residual unfiltered acid culture were next inoculated into similar sterile hay infusion tubes as controls. The fact that both *V. percolans* and *E. prodigiosus* uniformly grew out in the latter tubes proved that the culture had not been

sterilized by the short acid exposure. The second half of the original culture, with motility slightly if at all impaired, was then passed through the same filter used for the acid filtration, and the filtrate inoculated into the sterile hay infusion tubes; *V. percolans* regularly grew out of these tubes. *E. prodigiosus* was regularly absent in all filtrate and filtrate-inoculated tubes.

The filters used in the experiments of this paper were Berkefeld "V" (No. 3, $2\frac{1}{2}$ inches long by $\frac{5}{8}$ inch in diameter) with wall thickness of approximately 4 mm. The filtration lasted only two to six minutes, as shown in the tables. Twenty-five to 60 cc. of filtrate were drawn through the candle for each experiment. The unfiltered culture was invariably turbid and the filtrate clear; several examinations of filtrate specimens under the dark field microscope immediately after filtration did not serve to detect vibrios, though these ordinarily grew out in twenty-four hours or less when the filtrate was positive.

From all analogy it seems certain that the acidification of the culture decreased the potential difference at the filter-fluid and bacteria-fluid interfaces. The fact that seven experiments in which motility was stopped with acid gave clearly negative filtrates, whereas when motility was inhibited by other agencies (see below) only five cultures showed complete suppression and five partial suppression of filterability, may possibly be of significance; decreasing the charges on bacteria and filter wall may have allowed the molecular adhesion, "stickiness," to act more effectively in holding the bacteria to the filter capillary walls. This suggestion is advanced at this time, however, purely as a speculation.

As to possible changes in the size of the organisms with acidification of the media, none was ever observed under the dark field, though a more sensitive measure than mere inspection might with profit be employed. However we would expect such a change if it occurred to have been in the direction of a shrinking rather than a swelling, as acid was added and the organisms were thereby brought toward their point of zero potential difference against the medium (Loeb, 1922) just as hydration of gelatin particles decreases as they are brought toward their isoelectric point.

SUPPRESSION OF FILTERABILITY OF *V. PERCOLANS* BY NARCOTICS

The coincidence of the H-ion concentration at which the motility of *V. percolans* ceased with that at which filterability was lost made it desirable to stop motility by other means. This was done with narcotics, and filterability was again found to be coincidentally suppressed.

For each pair of experiments an actively motile culture was as before divided into two halves; one-half was shaken with a small amount of ether or chloroform and allowed to stand until dark field examination showed that locomotion had practically ceased. This was then filtered, the filtrate inoculated into sterile hay infusion tubes and other tubes inoculated with the unfiltered narcotised culture as a control of its viability. The untreated half of the original culture was then passed through the same filter and the filtrate inoculated into sterile tubes.

The residue of the narcotised unfiltered culture was allowed to stand in open cylindrical vessels until the anaesthetic had evaporated. Subsequent dark field examination showed the culture swarming with motile vibrios indicating, as did the inoculated control experiments mentioned above, that the chloroform and ether narcotised but did not kill the majority of the vibrios.

SUPPRESSION OF FILTERABILITY OF *V. PERCOLANS* BY CHILLING

Finally confirmation of the belief that suppression of the motility of the vibrio could inhibit its filtration through Berkefeld V candles was obtained by chilling the cultures. For each pair of experiments an actively motile culture was put in the ice box surrounded by ice water, and with melting ice in the culture vessels themselves. The filter was also chilled. One half of the culture was filtered at about 0°C.; the other half was allowed to warm for from twenty odd to seventy odd minutes and was then passed through the same filter. Vibrios regularly appeared in tubes inoculated with the latter filtrate, but were absent in some or all of the tubes inoculated with the filtrate from the filtration at 0°. Control inoculations of the culture at 0° into

TABLE 1
Effect of reaction of the medium on filtration of *V. percolans*

EXPERIMENT NUMBER	FILTER NUMBER	H-ION CONCENTRATION	MOTILITY	FILTRATION TIME minutes	FILTRATION PRESSURE mm. Hg.	RESULT	REMARKS
V ₁	1	6×10^{-6}	Almost stopped	6	0-286	-	Na-acetate acetic acid buffer used
V ₂	1	1×10^{-6}	Normal	5	0-295	+	<i>E. prodigiosus</i> control showed filter tight
V ₃	1	1×10^{-7}	Practically stopped	4	0-155	-	CO ₂ bubbled out in this and later experiments
V ₄	1	$\text{Ca. } 1 \times 10^{-7}$	Normal	4½	0-195	+	New filter used
V ₅	2	7.4×10^{-6}	Little if at all impaired	3½	0-225	+	Acid culture from V ₁ rendered alkaline and filtered
V ₆	2	7.9×10^{-6}	Little if at all impaired	3	0-245	+	
V ₇	2	Not acidified	Normal	3-4	0-245	+	
V ₈	2	Not acidified	Normal	7	0-145	+	
V ₉	2	Not acidified	Normal	3	0-235	+	<i>E. prodigiosus</i> control showed filter tight
*{V ₁₀ V ₁₁ }	3 3	7.2×10^{-6} 9.5×10^{-7}	Impaired Normal	3½ 4	0-255 0-225	- +	New filter used <i>E. prodigiosus</i> control showed filter tight
V ₁₂	Vibrios apparently killed by twenty odd minutes exposure to H-ion concentration about 1.5×10^{-6} . No growth in control tubes						
V ₁₃	3	3×10^{-6}	Normal	2½	0-195	+	<i>E. prodigiosus</i> control showed filter tight
{V ₁₄ V ₁₅ }	3 3	$1.2-1.3 \times 10^{-6}$ 2×10^{-6}	Much impaired Little if at all impaired	2½ 5½	0-245 0-245	- +	<i>E. prodigiosus</i> control showed filter tight

$\left\{ \begin{array}{l} V_{16} \\ V_{17} \end{array} \right\}$	4 4	1.6×10^{-6} 3.6×10^{-6}	Much impaired Little if at all impaired	6½ 5	0-109 0-109	- +	New filter used <i>E. prodigiosus</i> control showed filter tight
$\left\{ \begin{array}{l} V_{18} \\ V_{19} \end{array} \right\}$	4 4	1.7×10^{-6} 3.2×10^{-6}	Practically stopped Slightly impaired	3½ 3½	0-225 0-225	- +	
V_{20}	Results inconclusive because of bad culture medium						
V_{21}	Results inconclusive because of bad culture medium						
$\left\{ \begin{array}{l} V_{22} \\ V_{23} \end{array} \right\}$	5 5	4.5×10^{-6} 3.2×10^{-6}	Practically stopped Little if at all impaired	3½ 2	0-145 0-145	- +	<i>E. prodigiosus</i> control showed filter tight

* Experiments bracketed performed with two halves of same culture as above described.

TABLE 2
Effect of narcotics on filtration of *V. percolans*

EXPERIMENT NUMBER	FILTER NUMBER	APPROXIMATE PER CENT NARCOTIC ADDED*	MOTILITY	FILTRATION TIME minutes	FILTRATION PRESSURE mm. Hg.	RESULT	REMARKS
{ V ₂₆ V ₂₇	3	Chloroform 0.28 per cent added; not all dissolved	Much reduced	2½	0-200	Negative all tubes	Filter had been cleaned with Na ₂ CO ₃ and water since using for V ₁₅
	3	Untreated	Active	2½	0-180	Positive 4 tubes, negative 1 tube	
{ V ₂₈ V ₂₉	3	Ether 2.7 per cent	Practically stopped	5½	0-100	Negative all tubes	<i>E. prodigiosus</i> control showed filter tight
	3	Untreated	Active		0-105	Positive all tubes	
{ V ₃₀ V ₃₁	2	Ether 6.5 per cent added	Practically stopped	3½	0-220	Negative all tubes	<i>E. prodigiosus</i> control showed filter tight
	2	Untreated	Active	3	0-220	Positive all tubes	
{ V ₃₂ V ₃₃	5	Ether 3.9 per cent added	Practically stopped	2	0-270	Negative 5 tubes, positive 1 tube	<i>E. prodigiosus</i> control showed filter tight
	5	Untreated	Active	2	0-270	Positive all tubes	
{ V ₃₄ V ₃₅	3	Ether 3.2 per cent added	Greatly reduced	3½	0-335	Negative 3 tubes, positive 2 tubes	<i>E. prodigiosus</i> control showed filter tight
	3	Untreated	Active	3½	0-335	Positive all tubes	

* The percentage of narcotic tabulated is probably somewhat greater than that actually present in the cultures because of partial evaporation during additions of the ether and examinations of the cultures.

sterile hay infusion tubes at room temperature gave uniformly positive results.

The organisms regained active motility with surprising quickness on rewarming after chilling. Preparations could scarcely be got on the dark field microscope without showing motility, and within a few seconds locomotion was active.

ATTEMPTS TO FILTER VIBRIO PERCOLANS THROUGH N AND VIBRIO COMMA THROUGH V CANDLES

Inspection under the dark field microscope of living cultures of *V. percolans* and of *V. comma* impresses the observer with the general similarity of the two organisms but also with two peculiarities of *V. percolans* which are doubtless significant in determining filterability. These are, first, the frequency of highly motile, small, almost coccoid forms in *V. percolans* cultures, and, second the greater slenderness of *V. percolans*. Filar micrometer measurement has given as the average diameter of one hundred each of the organisms, *V. comma*, 0.46μ ; *V. percolans*, unselected, 0.35μ ; *V. percolans* (smaller individuals) 0.31μ , and *E. prodigiosus*, 0.31μ . The range of diameters in the *V. percolans* vibrios measured was 0.28μ to 0.51μ .

Doubtless in correlation with these characteristics is the fact that *V. percolans* under normal cultural conditions regularly gives a positive filtrate with Berkefeld V candles (though uniformly negative in six attempts at passage under similar conditions through three N candles) whereas *V. cholerae* is not a filter passer. Four unsuccessful attempts were made to pass *V. comma* through three Berkefeld Vs which had been shown before and after the attempted *V. comma* passage to be pervious to *V. percolans*.

DETECTION OF V PERCOLANS IN GROUND DOWN FILTER SECTIONS

Berkefeld filters, as is well known, are made of baked kieselguhr (diatomaceous earth). By microscopic examination of scrapings from a dry filter the fine irregular silicious granules

TABLE 3
Effect of chilling on filtration of *V. percolans*

EXPERIMENT NUMBER	FILTER NUMBER	PREPARATION	MOTILITY	FILTRATION TIME minutes	FILTRATION PRES-SURE mm. Hg.	RESULT	REMARKS
{ V ₃₆ V ₃₇	6	Chilled	Greatly reduced Active	2½	0-312	Negative all tubes	
	6	Chilled and rewarmed		2½	0-310	Positive all tubes	
{ V ₃₈ V ₃₉	6	Chilled	Much reduced	3½	0-240	Negative 4 tubes, positive 1 tube	<i>E. prodigiosus</i> control showed filter tight
	6	Chilled and rewarmed	Active	3½	0-240	Positive all tubes	
{ V ₄₀ V ₄₁	7	Chilled	Much reduced Active	3½	0-300	Negative all tubes	<i>E. prodigiosus</i> control showed filter tight
	7	Chilled and rewarmed		2½	0-250	Positive all tubes	
{ V ₄₂ V ₄₃	8	Chilled	Reduced	4½	0-355	Negative 2 tubes, positive 3 tubes	
	8	Chilled and rewarmed	Active	1½	0-342	Positive all tubes	
{ V ₄₄ V ₄₅	7	Chilled	Reduced	6	0-400	Negative 5 tubes, positive 1 tube	
	7	Chilled and rewarmed	Active	3½	0-395	Positive 4 tubes, negative 1 tube	

may be seen. Study of filter sections ground down to microscopic thinness shows a matrix of the fine granules containing at intervals spaces or lacunae of much greater size, i.e., a few to more than a hundred micra. For photomicrographs, see von Esmarch (1902) and Hofstädter (1905).

It has been the judgment of most authors who have considered the point that these lacunae could not afford a continuous passage through the filter wall and that a part, and of course a critical part, of the path of particles traversing the filter must be through the matrix or actual granular filter substance itself. This view has not been universally accepted, however, so that it seemed necessary to study the distribution of the vibrios in the filter.

A used V filter, no. 8, was again immersed in a vibrio culture and a filtration pressure maintained for a few minutes. It is to be emphasized that the bacteria were carried into the filter by filtration of only a few minutes duration and with filtration pressure of a few hundred millimeters of mercury. They were not allowed to "grow" into the filter. Absolute alcohol was drawn through to fix the freshly deposited vibrios. Fuchsin (acid) was drawn through for several hours in an attempt to stain the organisms satisfactorily; this later proved to have been unsuccessful. The filter was dehydrated and balsam in xylol was drawn through by suction and then allowed to seep through for a day or more. The balsam-impregnated filter was allowed to harden for a couple of weeks in the incubator. Rings were then cut with a knife and ground down with a coarse stone. A ring was mounted in balsam on a glass slide and final reduction to microscopic thinness carried out by prolonged grindings on fine stones. The balsam was removed by soaking in xylol, the xylol allowed to evaporate, and the organisms stained *in situ* with carbol fuchsin (acid) or methylene blue. The filter fragment was finally washed, dried and mounted in xylol balsam and studied with oil immersion.

The result is quite conclusive (see plate 2). The organisms may be seen in a stained coagulum on the surface and extending down into the superficial lacunae, and in among the granules of the matrix. By focussing with the fine adjustment the trans-

parent granules may be brought into focus above and below the vibrios, proving the latter to be in the capillary interstices between the silicious grains of the actual filter substance.

In plate 2 the organisms are readily discernible, a few in focus, others below and above the focal plane. No lacunae are shown in the photomicrograph, though they are present in other fields of the section. Intense illumination was used for the photomicrograph in order to give definition to the vibrios and the detail of the filter structure was thereby sacrificed. A few silica granules can, however, be made out.

THE INTERGRANULAR DIAMETERS AND RELATIVE POROSITY OF DIFFERENT BERKEFELD FILTER TYPES

The pore size of a number of Berkefeld filters has been estimated by the methods of Bechhold (1908). Compressed air was forced through the filters and the pressure in millimeters of mercury at which streams of bubbles began to issue from six or more pores was substituted as p in the formula:

$$D = \frac{4\beta \times 760}{p \times 1.933 \times 10^5}$$

β is the capillary constant of water at the temperature of the determination. D is the diameter in millimeters of the narrowest portion of the pores from which the bubbles emerge, i.e., it is an estimate of the average value of the spaces between the granules of the actual filter substance.

By using a small escape valve in the air line and a mercury manometer air pressure could be maintained within a few millimeters of any desired level. The values given in table 4 are in each case the average of several observations; they agree within a few mm. for any clean filter, but in used filters may vary by as much as 40 mm. Hg.

The variations in the mean values for the three types of filters, V. N. and W, is seen to be small; moreover it is in the wrong direction, i.e., the V's or most porous, appear to have the smallest intergranular spaces. These differences are thus probably fortuitous.

It may be pointed out that some of the used filters whose pores had been contaminated with dyes and proteins emitted bubbles at much lower pressures than the new ones, apparently indicating larger intergranular diameters. A similar effect was produced by mixing alcohol with the distilled water wetting the filters. It is thus evident that Bechhold's formula gives too high values for pore size in the presence of substances lowering the surface tension of the water in the pores.

TABLE 4

FILTER	SIZE	CONDITION	PRESSURE	CALCULATED DIAMETER	AVERAGE DIAMETER FOR FILTER TYPE
	<i>inches</i>		<i>mm. Hg</i>	μ	μ
V (10)	$2\frac{1}{8} \times \frac{1}{8}$	New	520	0.414	0.38
V (11)	$2\frac{1}{8} \times \frac{1}{8}$	New	567	0.380	
V (16)	$2\frac{1}{8} \times \frac{1}{8}$	New	585	0.373	
V (17)	$2\frac{1}{8} \times \frac{1}{8}$	New	581	0.376	
V (18)	$2\frac{1}{8} \times \frac{1}{8}$	New	588	0.371	
N (X)	$2\frac{1}{8} \times \frac{1}{8}$	New	466.5	0.461	0.45
N (XI)	$2\frac{1}{8} \times \frac{1}{8}$	New	493	0.437	
W (1)	$2\frac{1}{8} \times \frac{1}{8}$	New	410.5	0.524	0.43
W (2)	$2\frac{1}{8} \times \frac{1}{8}$	New	450	0.478	
W (X)	$2\frac{1}{2} \times 1$	New	592	0.369	
W (XI)	$2\frac{1}{2} \times 1$	New	636	0.343	
V (6)	$2\frac{1}{8} \times \frac{1}{8}$	Used	588	0.366	
N (1)	$2\frac{1}{8} \times \frac{1}{8}$	Used	617.5	0.349	
N (2)	$2\frac{1}{8} \times \frac{1}{8}$	Used	468	0.460	
N (VIII)	$2\frac{1}{2} \times 1$	Used	287	0.761	
W (IX)	$2\frac{1}{2} \times 1$	Used	307.5	0.710	

Tested by the second method, namely that of determining the rate of flow of distilled water through the filters as a function of the pressure head, the filters of course showed themselves more porous in the order $V > N > W$. Plotting pressure against rate of flow, the points fall along straight lines passing very nearly through the origin. The slopes indicated the following rates of flow for three new $2\frac{1}{8} \times \frac{1}{8}$ inches filters:

V (10), 0.45 cc. per second per 100 mm. Hg pressure head
N (X), 0.21 cc. per second per 100 mm. Hg pressure head
W (1), 0.125 cc. per second per 100 mm. Hg pressure head

The filters used thus evidently differed in the number and size of the gross pores or lacunae rather than in the size of the intergranular spaces.

With the foregoing conclusion in mind further attempts were made to filter *V. percolans* through N filters, as follows:

Approximate volume filtered, 150 cc.; filtration pressure, 0-400 mm. Hg;
result negative
Approximate volume filtered, 125 cc.; filtration pressure, 0-500 mm. Hg;
result negative
Approximate volume filtered, 575 cc.; filtration pressure, 0-700 mm. Hg;
result negative
Approximate volume filtered, 480 cc.; filtration pressure, 0-744 mm. Hg;
result negative

The passage of filterable organisms through the filter wall is evidently then greatly facilitated by the lacunae, even though one critical part of the passage has to be through the fine interstices of the granular filter substance.

DISCUSSION

Porous filters of the Berkefeld type, as of course is well known, have shown themselves of much practical utility in freeing solutions of thermolabile substances from bacteria, in separating the ordinary bacteria from the "filterable viruses" (Wolbach, 1912), in purifying water, and in other ways. The majority of these filters under the usual conditions of short duration of filtration and moderate pressure give filtrates sterile for most known microorganisms. However, the familiar fact that the microorganisms penetrate the filter if contact of the culture with it is sufficiently long-continued, especially if suitable nutrient material is at hand in the filter pores (Hofstädter, 1905), would seem to indicate that the filter owes its tightness to the tortuosity and length of the passage to be traversed as well as to its narrowness, rather than to an absolute excess in all cases of the smallest dimensions of the microorganisms over the smallest diameters of the pores they must pass through.

Adhesion also doubtless plays a part in holding back the bacteria (Bechhold, 1918). In dark field examination of cultures of *V. percolans* in films between cover-glass and slide the vibrios are found adherent to the upper or lower glass surfaces in increasing numbers as time goes on. Often a vibrio can be seen to swim against the cover glass and stick there; occasionally an organism will stick to the cover glass only at one point and thresh about in the medium with this contact as a fixed point. With *E. prodigiosus* this stickiness doubtless plays even a more considerable part, for colonies on solid agar are extremely adherent and when scraped with a platinum loop come off in long strings. A fluid culture of *E. prodigiosus* can be distinguished from one of *V. percolans* also by the stringing out of the former when it is filtered through cheese-cloth or even when a platinum loop is withdrawn from the prodigiosus culture.

The "effective pore size" of a bacteria-tight filter has been defined by Rosenthal (1908) as "the narrowest diameter which is present in each of the extraordinarily many porous passages, at least in one place; it becomes, if we consider layers of one and the same mass increasing regularly in thickness, smaller, at first rapidly, then more slowly." He estimates the effective pore size as between 0.5 and 2μ .

Schmidt (1910) from the fact that he was able to filter *Ps. fluorescens-liquefaciens* but not staphylococci and Gram-negative diplococci, whose diameter was about 0.8μ , gives 0.2μ and 0.8μ as upper and lower limits of the effective pore size of Berkefeld filters. "One will certainly not be far from the true value, if he assumes a mean value of about 0.5μ ." Assuming that the smallest quartz grains visible in ground-down sections of Berkefeld filters approximate close-packed spheres, he calculates the actual diameter of the finest pores between them as about 0.3 to 0.4μ .

Bechhold (1908) has calculated the diameter of pores of filters by the minimum pressure able to force air through. He gives the mean pore diameters of the larger pores of a new chamberland F filter as 0.23 to 0.41μ .

Our own estimates on Berkefeld filters are given above.

In view of these estimates it is of interest that under the circumstances of our filtrations motile *V. percolans* passes through Berkefeld V but not Berkefeld N candles and *V. comma* through neither. The measured diameters of one hundred unselected individuals of *V. percolans* vary from 0.28 to 0.51 μ , with an average value of 0.35 μ . Selecting the smaller individuals, the mean diameter found was 0.31 μ . The average diameter of one hundred unselected cholera vibrios was 0.46 μ .

The mechanics of the situation determining sterility or non-sterility of the filtrate from any given bacterial culture are evidently then such that under ordinary circumstances we may expect bacteria-free filtrates, but in the presence of any circumstances exceptionally favorable to passage through the filter, we anticipate a seeding of the filtrate. This expectation the literature shows indeed to be fulfilled.

The favoring circumstances may be small size alone e.g., the globoid bodies of poliomyelitis (Flexner and Noguchi, 1913), of 0.2 μ average diameter, the coccoid bodies described by Foster as a cause of coryza (1917), typically 0.2 to 0.3 μ in diameter, *Bacterium pneumosintes* (Olitsky and Gates, 1921, 1922), 0.15 to 0.3 μ in length, a half to a third as large in diameter and the minute Gram-negative anaerobes isolated from human throats by Olitsky and Gates (1922), and probably the coccoid bodies described by MacCallum and Oppenheimer in vaccine lymph as about one-tenth the size of ordinary streptococci (1922).

Or small size and motility may combine to produce filterability, e.g., *Spirillum parvum* of v. Esmarch (1902), diameter 0.1 to 0.3 μ , and the filterable spiral organisms and the protozoan of tap water discovered by Borrel (1903):

Ceux qui passent le plus ordinairement sont des vibrios très polymorphes dont certains sont à la limite de la visibilité, reconnaissable à leur cil unique. Ces formes sont celles qui passent à travers les pores de la bougie, puis, dans la culture, il se développe des vibrios de dimensions variable, quoique cette culture paraisse tout à faire pure.

* Dans d'autre cas, ce sont des formes spirillaires qui passent et la culture montre des très longs filaments grêles, invisible à l'état frais, assez semblables aux spirilles de la fièvre récurrente, mais plus courts.

Dans ce même milieu, j'ai obtenu, par filtration, la culture pure d'éléments très particuliers, que je considère comme appartenant au groupe des protozoaires, et que j'ai désigné sous le nom de *Micromonas mesnili*. Ce sont très ordinairement des éléments ovoïdes allongés de $\frac{1}{2}\mu$ de largeur sur 3 à 4μ de longueur, munis de deux cils trepus, plus gros que des cils de bacteries, plus rigide . . . etc.

A clear case of the determinative value of motility is the passage of motile *V. percolans* through Berkefeld Vs and the failure to pass of non-motile percolans.

Slenderness, motility and flexibility may all be complementary factors in enabling organisms to pass the filter, e.g., the filterable spiral organisms of Wolbach and Binger, *Sp. elusa* (1914) and *Sp. biflexa* (1915), and *Leptospira icterohaemorrhagiae* (Noguchi 1919), diameter 0.25μ , and Noguchi's *Leptospira icteroides* (Noguchi, 1919), 0.2μ in diameter.

Again slenderness, motility and flexibility may not suffice and specially favorable pressure conditions may be require in addition, e.g. the experience of Todd and Wolbach (1914), who conclude that:

Sp. duttoni in an infective form can be forced through a Berkefeld filter by pressures of over 50 pounds to the square inch.

Sp. duttoni cannot be filtered through a Berkefeld filter in an infective form by atmospheric pressure.

Similarly Hofstädter, in an admirable work on the penetration of bacteria through the finest capillaries (1905), was able to force bacteria through capillaries under a filtration pressure of 50 to 100 atmospheres which would not permit their passage under pressures of two to three atmospheres or without a pressure head.

It is a pleasure to acknowledge the many courtesies of the late Prof. H. C. Ernst, of Profs. S. B. Wolbach and W. T. Bovie and assistance on a number of points of Mr. Shields Warren and Emily B. H. Mudd.

SUMMARY

Cultures of *Vibrio percolans*, grown in hay infusion whose reaction was approximately neutral, when brought to a hydrogen-

ion concentration of 5×10^{-6} (pH = 5.3) or above rapidly lose motility. Such non-motile cultures gave negative filtrates with Berkefeld V candles through which motile vibrios regularly passed.

Similarly the filterability of *V. percolans* under the conditions of our experiments was suppressed completely five times and partially five times by inhibiting motility with ether, with chloroform or by chilling.

In all cases the viability of the non-motile culture, at least for the duration of the experiment, was proved by positive subcultures. Growing with *V. percolans* in the cultures which were filtered was a feebly motile strain of *E. prodigiosus*. This organism did not appear in the filtrates whether positive or negative for *V. percolans*.

It is thus shown that when a culture is drawn through an irregular capillary bed under circumstances in which a few organisms may or may not pass through, motility may be a critical factor in determining passage. Motility doubtless aids passage both in purely mechanical ways and by combating the tendency of the organisms to adhere to the pore walls.

V. percolans did not in ten attempts made pass through Berkefeld N candles. Attempts to pass *V. comma* through three Berkefeld V's shown before and after to be pervious to *V. percolans* were likewise unsuccessful. *V. comma* repeatedly showed itself able to pass by its own powers of growth and locomotion through a layer of ten centimeters of quartz sand in shorter time than *V. percolans*. Its inability to traverse Berkefeld filters is therefore not referable to inferior motility but to slightly larger size than *V. percolans*. The average diameters found for cholera vibrios (unselected) was 0.46μ , for *percolans* (unselected), 0.35μ , for *V. percolans* (smaller individuals), 0.31μ , for *E. prodigiosus* (unselected), 0.31μ .

Estimation of the diameters of the intergranular spaces of Berkefeld filters by Bechhold's method of forcing air through gave the following average values:

V type, 0.38μ ; N type, 0.45μ ; W type, 0.43μ . The differences are interpreted as fortuitous.

Estimation of porosity by determining the rate of filtration of water at various pressures shows the V type the most and the W the least porous. The following filtration rates with distilled water for $2\frac{5}{8} \times \frac{5}{8}$ inch candles were obtained:

V, 0.45 cc. per sec. per 100 mm. Hg pressure head

N, 0.21 cc. per sec. per 100 mm. Hg pressure head

W, 0.125 cc. per sec. per 100 mm. Hg pressure head

The differences in porosity are then evidently a matter of the relative size and numbers of the gross pores rather than of the intergranular spaces.

Sections of a Berkefeld V candle used for filtration of *V. percolans* have been ground down to microscopic thinness and the vibrios stained and demonstrated in the interstices between the silicious granules of the filter substance. The intergranular diameters are therefore of critical importance in determining the penetrability of the filters by bacteria.

That the relative numbers and sizes of the gross pores or lacunae may also be of critical significance is shown by the successful passage of motile vibrios through V filters and their failure to pass N's.

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PLATE 1

FIG. 1. E. PRODIGIOSUS, TWENTY-FOUR HOUR AGAR CULTURE IN 0.5 PER CENT AGAR MOUNT

Five inches, 1.9 mm., 7 oc. \times 1120 diameters. Ultraviolet light, $\lambda = 2800$ Å, Mg electrodes.

FIG. 2. V. CHOLERAE, TWENTY-FOUR HOUR CULTURE

Conditions same as above

FIG. 3. V. PERCOLANS, TWENTY-FOUR HOUR CULTURE

Conditions same as above

Photomicrographs by Mr. Shields Warren

PLATE 2

GROUND DOWN SECTION OF BERKEFELD V FILTER WITH VIBRIO PERCOLANS STAINED IN SITU WITH METHYLENE BLUE

A few vibrios in focus, others below and above the focal plane. Darker zone along left margin of field deeply stained coagulum at surface of filter. Oil immersion. \times 1000 diameters. Photograph by Prof. S. B. Wolbach.



FIG. 1

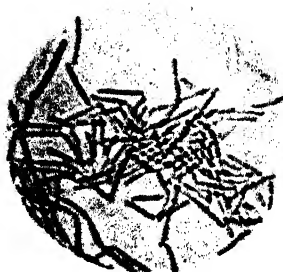


FIG. 2



FIG. 3

(Mudd: Penetration of bacterin.)



(Mudd: Penetration of bacteria.)

PHYSIOLOGICAL STUDY OF AZOTOBACTER CHROOCOCCUM. I

INFLUENCE OF VITAMINE B (?) AND NUCLEIC ACID ON AZOTOBACTER

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INTRODUCTION

This paper deals with a preliminary investigation in regard to the influence of vitamine B (?)¹ and phyto-nucleic acid² upon the growth and nitrogen-fixing power of *Azotobacter*,³ an investigation which was undertaken in conjunction with our major problem of soil fertility now in progress.

EXPERIMENTAL

The influence of different concentrations of vitamine B (?) and phyto-nucleic acid on the growth of Azotobacter

These substances were tested for their accessory action by adding them in varying concentrations to Ashby's solution of pH 7.2. First 1 per cent sterile solutions of these substances were made and varying amounts added to sterile, double concentrated Ashby's solution, in volume equivalent to one-half the final volume. Finally the solution was made up to the desired volume by adding sterile water. The medium, after being

¹ Vitamine B(?), yeast vitamine which was prepared by the Harris Laboratory, Tuckahoe, New York, and shown to contain water-soluble vitamine B, in highly concentrated form.

² Phyto-nucleic acid, prepared from yeast cells by the Will Corporation, Rochester, New York.

³ *Azotobacter*, A4 of Prof. D. H. Jones of O. A. C., Guelph, Canada.

tested for sterility, was inoculated with 0.05 cc. of a ten-day-old culture of *Azotobacter* in Ashby's solution.

Table 1 indicates that the addition of these substances at 1:10,000 or higher concentration seems to have a similar stimulating effect on growth. No difference in degree of stimulation in concentrations above 1:10,000 is apparent so far as the visual observation of turbidity is concerned.

TABLE 1

The influence of varying concentrations of vitamine B(?) and phyto-nucleic acid on the growth of Azotobacter

CONCENTRATIONS	GROWTH AFTER FIVE DAYS AT 25°C.	
	Vitamine B(?)	Phyto-nucleic acid
1:500	++	++
1:1,000	++	++
1:10,000	++	++
1:100,000	+	+
1:1,000,000	+	+
Ashby solution (plain)	+	+

Rate of acceleration of growth

An attempt was made to record the rate of acceleration of growth by these two substances in 1:10,000 concentration.

From table 2 it is apparent that the stimulation is manifest within forty-eight hours, and is very marked at the fifth day. Here again it may be noted that the two substances studied are behaving very similarly.

TABLE 2

Rate of acceleration of growth

MEDIA	24 HOURS	48 HOURS	96 HOURS	120 HOURS	240 HOURS	360 HOURS
Ashby + vitamine B(?).....	—	+	+	++	++	++
Ashby + phyto-nucleic acid.....	—	+	+	++	++	++
Plain Ashby.....	—	—	+	+	+	+

+, moderate visible growth; ++, indicates abundant visible growth.

Quantitative determination of growth of Azotobacter and nitrogen fixed

At the end of ten days, the number of organisms was counted by plating on Ashby's agar and the total nitrogen was determined.

Table 3 indicates the quantitative increase in the number of Azotobacter cells and the amount of nitrogen fixed. It is apparent that there is a slight difference in the stimulation by vitamine B (?) and phyto-nucleic acid, the former appearing to be more effective than the latter. Also it is interesting to note the parallelism between the growth and the total amount of nitrogen fixed.

TABLE 3
Growth of Azotobacter and nitrogen fixed

MEDIA	NUMBER OF ORGANISMS PER CUBIC CENTIMETER		N PER 100 CC.		GAINED
	Initial	After ten days	Initial N	After ten days	
		<i>millions</i>	<i>mgm.</i>	<i>mgm.</i>	
Ashby + vitamine B(?).....	19,000	1,550	0.2	15.0	14.8
Ashby + phytonucleic acid.....	19,000	1,250	0.2	13.0	12.8
Plain Ashby.....	19,000	450	0.2—	5.7	5.5

DISCUSSION

The vitamine B (?) used in the investigation was prepared by the Harris Laboratory and was claimed to be a highly concentrated vitamine, water-soluble-B (fraction II) described by Osborne and Wakeman.⁴ It is stated that the fraction II contains 7.5 per cent of nitrogen, or 4.46 per cent of the total yeast nitrogen, or 31.8 per cent of the nitrogen of yeast extract. Consequently the concentration of 1:10,000 which was used in the investigation contained a very small amount of nitrogen, approximately 0.0075 mgm.

The influence of this preparation on *Saccharomyces cerevisiae* was determined by the author as well as by his students in the physiology class and an accessory effect similar to that reported

⁴ Jour. Biol. Chem., 1919, vol. 40, no. 2, 383.

by Bachman⁵ was observed. Recently animal tests on this preparation were carried out by Bailey and his co-workers⁶ and a marked accessory influence was noted.

The phyto-nucleic acid (1:10,000) which was used in this investigation contains approximately 0.016 mgm. of nitrogen.

When the experimental data recorded previously are considered in the light of the facts stated, it seems to be apparent that these two substances exert an accessory influence other than the supply of a very small quantity of food substance.

It is not the purpose of this paper to convey the idea that vitamine B (?) and phyto-nucleic acid are similar in substance. The experimental data, however, indicate plainly that both of these substances have a stimulating influence on *Azotobacter chroococcum*.

CONCLUSIONS

The water-soluble-B (?) vitamine and phyto-nucleic acid exert marked stimulating influence on *Azotobacter* A4 in regard to its growth and fixation of nitrogen.

⁵ Ibid., 1919, vol. 39, no. 2, 235.

⁶ Bull. 240, Connecticut Agr. Exper. Station, 1922, 44-47.

THE ENZYMIC CONTENT OF BACTERIAL SPORES¹

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That bacterial spores are lacking in enzymic activity is a natural deduction from the fact that the spore is a resting stage in the life history of the organism. However, the work of the Kopeloffs² on the demonstration of active enzymes in mold spores suggests the possibility of active enzymes in bacterial spores. It is the purpose of this paper to record some preliminary experiments on the possibility of demonstrating active enzymes in bacterial spores. After our work was begun the following statement was found in Effront's "Biochemical Catalysts in Life and Industry" (p. 312):³

In this connection we must also take into consideration the observation of Effront, according to which the bacterial spores, attenuated either by an antiseptic or by heat, show themselves the more productive of enzymes the more difficult their germination. Under certain conditions and in the presence of antiseptics the spores may produce an intense secretion of enzyme in a liquid without, however, arriving at germination.

Up to the present time we have been unable to obtain Effront's original paper and so we do not know what data he had to support his statement.⁴

¹ Published by the Permission of the Director of the Michigan Agricultural College Experiment Station.

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² "Do mold spores contain enzymes?" M. Kopeloff and L. Kopeloff, *Jour. Agric. Research*, 1919, **18**, 195-209.

³ "Biochemical Catalysts in Life and Industry." Jean Effront. Trans. by S. C. Prescott. First Ed., John Wiley and Sons, 1917.

⁴ Between the time of submitting this paper for publication and the reading of the proof Effront's original article ("Sur l'Action Chimique des Spores," Jean Effront. *Le Moniteur Scientifique*, Queensville, Feb., 1907, Liv. 782, pp. 81-87) has been found and studied; his data agree in every respect with ours.

In the present work suspensions of washed spores were added to certain substrates in the presence of antiseptics and observations and chemical determinations were made for the purpose of detecting chemical changes in the substrates.

Twelve cultures of aerobic, spore-forming bacteria were used. The names of the species will not be given at the present time because a positive identification has not been made. The cultures were purified by plating and then sown upon the surface of agar in Kolle flasks and allowed to incubate for five and one-half months at room temperature under a bell jar. The growths were then scraped off with a sterile, bent glass rod and suspended in sterile physiological salt solution. These spore suspensions were centrifuged for forty to fifty minutes, which resulted in precipitating the spores and leaving the few vegetative rods in the supernatant liquid which was pipetted off. This process of washing was repeated a second time and then the spores were made into a uniform suspension in physiological salt solution. In this connection it is worthy of note that before the washings, the growth contained small numbers of vegetative rods which had failed to sporulate, but that after washing, such vegetative rods could not be found. Apparently the spores were appreciably heavier than the rods. Possibly also the latter were dead since these cultures were several months old. From 1 to 2 cc. of these spore suspensions were used to inoculate the various substrates.

The following is a description of the substrates used with the results secured with each substrate:

OXIDASE TESTS

1. Two cubic centimeters of spore suspension added to 10 drops tincture of guaiac (1 gram resin in 60 cc. of 95 per cent alcohol). All negative.

2. Two cubic centimeters of spore suspension added to 10 drops of a 1 per cent alcoholic α -naphthol solution. All negative.

3. Two cubic centimeters of spore suspension added to 10 drops of a 1 per cent aqueous solution of p-phenylene-diamine hydrochloride. All negative.

4. Indophenol test on 2 cc. of spore suspension. All were slightly positive, while controls were negative.

REDUCTASE TESTS

One-half cubic centimeter of a methylene blue solution (5 cc. saturated alcoholic solution of methylene blue + 195 cc. of water) was added to 10 cc. water. Added 1 cc. of spore suspension. All tubes negative after five hours at 45 to 48°C.

CATALASE TESTS

A sterile fermentation tube was filled with 1 per cent hydrogen peroxide. Two cubic centimeters of spore suspension were added. All 12 organisms produced an evolution of gas, 4 of them being very active. Boiling of the spore suspension greatly reduced the activity, though in no case was it prevented entirely.

LIPASE TESTS

One cubic centimeter of the spore suspension was added to 100 cc. of a butterfat emulsion (made by emulsifying pure butterfat with gum acacia and water, adding 1 per cent formalin and sterilizing in flasks in the autoclave). After incubating for two weeks in a dark room, the acidity in 10 cc. of an uninoculated control was compared with that in the inoculated flasks. To 10cc. of the emulsion was added 50 cc. of neutralized water and then 50 cc. of a neutralized mixture of alcohol-ether (1:1). One-tenth normal alcoholic potassium hydroxide was used for titration and rosolic acid was used as the indicator. Eleven of the cultures were clearly negative, while one of them gave an increase of 0.4 cc. of tenth normal potassium hydroxide over the control (0.8 for control, 1.2 for culture).

CASEINASE TESTS

To 350 cc. of sterile skim milk was added 2 cc. of the spore suspension, enough chloroform to form a distinct layer on the bottom and enough toluene to form a $\frac{1}{4}$ inch layer on top. The flasks were then incubated at room temperature for periods varying in the different tests from two to four weeks. Suitable controls were also incubated. Examinations by the Breed method

were made at the beginning and from time to time during the periods of incubation to see if the spores would germinate.

The controls and the cultures were then examined chemically for hydrolysis of the casein by Sorensen's Formol Titration Method, after first precipitating the casein and removing ammonia by aeration. The details of the process will not be given at present since some modification will be necessary to get entirely satisfactory results. There were some indications of hydrolysis of the casein in 3 cases, in one of which the hydrolysis was marked. However, it is not certain that the hydrolysis was produced by caseinase, since the method as conducted gave some opportunity for chemical hydrolysis.

GELATINASE TESTS

Only four cultures were tested for gelatinase. Tubes of neutral, sterile, solidified 0.5 per cent phenol-gelatin were inoculated on the surface with 1 cc. of the spore suspension. Two to 3 cc. of toluene were placed on top of the suspension and the tubes incubated for seventeen days. Liquefaction of the gelatin was observed as follows:

	<i>per cent</i>
Culture 1.....	25
Culture 2.....	13
Culture 3.....	6
Culture 4.....	3

Smears were made of the suspensions and of the liquefied portions of the gelatin and examined carefully for spores and bacilli. No bacilli were observed but the spores were numerous. None of them showed evidences of germination. A test for amino acids by Sorensen's Formol Titration Method was also conducted as follows:

The gelatin was melted and the contents thoroughly mixed by agitation of the tube. Five cubic centimeters were added to some distilled water and the whole brought to neutrality to phenolphthalein. Ten cubic centimeters of neutralized formalin were then added and the acidity titrated with tenth normal potas-

sium hydroxide, using phenolphthalein as indicator. The following results were secured:

	<i>cc. N/10 K.H</i>
Control (uninoculated).....	0.0
Culture 1.....	1.5
Culture 2.....	0.7
Culture 3.....	0.4
Culture 4.....	0.3

Both of these tests clearly prove that gelatinase was present even though germination did not take place.

From the foregoing experiments the tentative conclusion seems to be justified that bacterial spores do exhibit some enzymic activity even when there is no evidence of germination. If further experiments substantiate the above preliminary tests we may have to modify some of our ideas of spores. Aside from the theoretical interest of the question, it has some practical bearing on the preservation of foods and possibly also on the rôle of the spores in the soil.

UTILIZATION OF THE SALTS OF ORGANIC ACIDS BY THE COLON-AEROGENES GROUP

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The sanitary importance of the colon bacillus and its allies has led to numerous attempts to classify this complex group of bacteria, for the possible existence of certain types within the group which might be of especial sanitary significance has long been recognized. Among the various biochemical tests usually applied to the study of bacterial groups, the qualitative fermentation of carbohydrates and higher alcohols has for many years held the most prominent place and the list of the substances so employed has been an ever lengthening one as new products have been made available. The list of non-nitrogenous carbon-containing compounds which may be utilized as sources of energy need not be limited, however, to the commoner carbohydrates and higher alcohols since it is known that some microorganisms possess the ability to break down a great variety of carbon-containing substances of diverse structure.

The present investigation was undertaken as a study in bacterial nutrition to gain some idea of the utilization of the salts of the commoner organic acids by members of the colon-aerogenes group. Furthermore, should any separation of the various types within the group be effected, it was desired to determine whether this might be correlated with the distribution of these organisms in nature and therefore offer a possibility of practical use in the estimation of the sanitary quality of water.

There are many scattered references in the literature on the utilization of organic acids, or their salts, by various bacteria, and some attempts have been made to formulate systems of classifi-

cation based upon the fermentation of these substances. In spite of these suggestions, however, the organic acids have not found the general use to which they are seemingly entitled in the differentiation and classification of bacterial types. In 1892 Van Ermengem and Van Laer reported that the colon bacillus was able to break down formic, succinic, citric and tartaric acids with the formation of hydrogen, carbon dioxide and methane. Maassen (1895) endeavored to study the utilization of many of the commoner organic acids by adding them to a basic medium containing pepton and certain inorganic salts. The decomposition of the organic acids was indicated roughly by the development of an alkaline reaction and a greater turbidity than was shown by the same cultures in plain broth controls. Capaldi and Proskauer (1896) employed the ammonium salts of many organic acids in synthetic media which were used in a comparative study of *Bact. coli* and *Bact. typhosum*.

Much of the subsequent literature seems to have centered around the nutritive requirements and the products of metabolism of the colon bacillus and other members of the colon-typhoid group. Harden (1901), while studying the fermentation of carbohydrates and allied compounds by *Bact. coli-communis*, noted that formates were decomposed. Pakes and Jollyman (1901) studied the decomposition of sodium formate by various bacteria. Among the organisms capable of attacking formate were *Bact. coli-communis*, *Bact. lactis-aerogenes*, *Bact. cloacae* and *Bact. enteritidis*. It was estimated that 25 to 30 per cent of the formate was destroyed when 0.2 to 2 per cent was employed in the culture media. Grey (1913-1914) recognized that formic acid was an intermediate product in the decomposition of glucose by *Bact. coli* and that the formic acid itself was attacked in a secondary fermentation. Omelianski (1907) proposed the use of sodium formate broth to differentiate certain types. *Bact. coli*, a "Pseudodiphtheria bacillus" and the paratyphoids A and B attacked the formate with alkali production, while *Bact. typhosum* and *C. diphtheriae* lacked this property. Karczag and Móczár (1913, 1915) found that pyrroacemic acid was attacked by *Bact. coli*, *Bact. paratyphosum* B, *Bact. enteritidis* and *Bact. typhi-murium*. Gas was

formed from the pyrrolic acid. Oxalacetic acid was attacked only very slightly by those organisms which possessed the property of decomposing pyrrolic acid (Karczag and Breuer, 1915). On the other hand, the above organisms, together with *Bact. pneumoniae*, produced a "hydrogen fermentation" from formic and glycolic acids (Karczag and Schiff, 1915). In the course of a study of several atypical *Bact. paratyphosum* B. cultures, Wagner (1913) reported that in plain nutrient broth plus 1 per cent pyrrolic acid an abundant gas formation was produced by typical *Bact. paratyphosum* B, *Bact. enteritidis* and *Bact. coli*. Altobelli (1914) reported that *Bact. coli* was capable of attacking the sodium salts of malic, tartaric and citric acids and converting them into carbonates. *Bact. typhosum* did not possess this property in the great majority of cases. Observations on the use of the above organic acid salts were extended to other bacteria.

More recently, several publications have served to emphasize again the utilization of organic acids. Braun and Cahn-Bronner (1921a) found that *Bact. paratyphosum* B was capable of growing readily in a chemically definite medium in which ammonium lactate formed the sole source of both nitrogen and carbon. Furthermore, the organism was carried through 100 successive passages in this medium in the course of one and a half years. The lactic acid could be replaced by succinic or citric acids but not by carbonates, formates or acetates. The same authors subsequently extended this work (1921b) to include *Bact. coli* and a number of other types. *Bact. coli* was able to develop through continuous passages in the lactate medium. Wagner (1920) employed a number of organic acids in an investigation of the biochemistry of the colon-typhoid group. The potassium or sodium salts of the acids were added to ordinary broth or agar and because of this it is difficult to interpret his results, although he evidently obtained undoubted utilization of certain of these organic acid salts by *Bact. coli* and *Bact. paratyphosum* A and B. Brown (1921), in connection with a study of the use of sodium citrate solution for collecting blood samples for cultural tests, made a series of observations on the growth of numerous species of bacteria, mostly pathogens, in plain broth containing 1 per cent sodium citrate. Upon compari-

son with plain broth controls it was found that some species grew more luxuriantly in the citrate medium while the growth of others was inhibited. Addition of a lead acetate solution to forty-eight hour cultures produced a voluminous white precipitate in the uninoculated control tubes and in those cultures exhibiting inhibition of growth. Where an increased luxuriance of the culture was evident there was little precipitate and in these cases Brown assumed that the citrate had been broken down and used by the organism. In the list of cultures which he tested in the citrate broth it is noteworthy that *Bact. lactis-aerogenes* and *Bact. cloacae* showed the increased luxuriance of growth while the growth of *Bact coli* was inhibited. Taken in connection with the lead acetate test, we have here a suggestion that the aerogenes-cloacae types are capable of utilizing citrate while coli is not. Apparently the significance of this observation as applied to differentiation of the colon-aerogenes group and its possible application to sanitary water analysis were overlooked, for this promising opening was not followed.

Pesch (1921) made a study of selective media for the colon-aerogenes group and used several synthetic combinations in which either tartrate or citrate was supplied as the only source of carbon. *Bact. paratyphosum* B developed readily in either medium, *Bact. enteritidis* usually exhibited slight growth, 5 *Bact. coli* strains showed abundant growth in tartrate but refused to grow in the citrate medium, while *Bact. paratyphosum* A and *Bact. typhosum* refused to develop in any case. When he repeated the results in the citrate medium with 15 additional colon strains from urine and feces these also failed to grow.

In this country the work of Ayers and his associates has advanced considerably our knowledge of the utilization of organic acids. Ayers and Rupp (1918) called attention to the destruction of organic acids which may occur in a glucose medium simultaneously with the sugar fermentation. The reversion to an alkaline reaction exhibited by some cultures is due largely to the oxidation of the salts of the organic acids to bicarbonates and carbonates. Ayers, Rupp and Johnson (1919) used organic acid salts in a study of the classification of alkali-forming bacteria which do not attack the carbohydrates ordinarily employed in fermentation tests. As

a result of their work they suggest that since organic acids are suitable sources of carbon for many bacteria, they might be used as an extensive set of test substances in studies of the biochemical properties of microorganisms.

EXPERIMENTAL

From the foregoing citations it is evident that the organic acids offer a promising field for studies of the nutrition and metabolism of various groups of bacteria and at the same time may prove to be of considerable value in classification. Following this idea, it decided to determine the availability of a number of these acids as sources of carbon for members of the colon-aerogenes group. For this purpose a simple culture medium was adopted which would contain the organic acids as the only source of carbon. The basal medium employed throughout this work consisted of:

Distilled water.....	1000 cc.
NaCl.....	5.0 grams
MgSO ₄ ·7H ₂ O.....	0.2 gram
(NH ₄)H ₂ PO ₄	1.0 gram
K ₂ HPO ₄	1.0 gram

This combination gives a colorless clear solution having a pH of 6.7 to 6.8. To this solution the various organic acids were added and the reaction brought back to pH 6.8 by the addition of normal sodium hydroxide solution. Ammonium phosphate was used to supply the necessary nitrogen for development. An inorganic nitrogen-containing salt must be employed, since all carbon is to be excluded from the basal medium and to be derived solely from the test substances added.

Several preliminary tests showed that when a readily available source of carbon was added to the above combination of inorganic salts an abundant growth of both *Bact. coli* and *Bact. aerogenes* resulted. When the available carbon-containing compound was omitted, no growth was apparent. This is shown in table 1. Evidently in the foregoing combination of inorganic salts we have all the essentials for development of the colon-aerogenes group with the exception of an available carbon-containing compound. By adding the different organic acids, one at a time, to this solu-

tion their availability may be determined by noting the resultant development of the cultures.

The foregoing combination of inorganic salts cannot be regarded as an ideal one. It was merely found that when available nitrogen and carbon were present it permitted a moderately rapid and luxuriant growth of the colon-aerogenes cultures. In an effort to simplify the medium magnesium sulphate was omitted. This resulted in a poor growth of all the *Bact. coli* and *Bact. aerogenes* cultures. The addition of 0.01 per cent of calcium chloride, either with or without magnesium sulphate, was tried but since no greater luxuriance of growth could be discerned in either case as a result of its addition it was not employed regularly. These results should not

TABLE 1

Showing the effect upon growth of the addition of an available source of carbon to the solution of inorganic salts

COMPOSITION OF MEDIUM	ORGANISM USED FOR INOCULATION	RESULTANT GROWTH
Inorganic salts.....	<i>Bact. coli</i>	None
Inorganic salts.....	<i>Bact. aerogenes</i>	None
Inorganic salts + 0.2 per cent glucose.....	<i>Bact. coli</i>	Abundant
Inorganic salts + 0.2 per cent glucose.....	<i>Bact. aerogenes</i>	Abundant
Inorganic salts + 0.2 per cent lactose.....	<i>Bact. coli</i>	Abundant
Inorganic salts + 0.2 per cent lactose.....	<i>Bact. aerogenes</i>	Abundant

be held as indicating that calcium is not needed for development, since traces at least might have been present as impurities in the other salts.

Experiments with various organic acids

A number of different organic acids were available for the present study. These were added to the basic solution of inorganic salts in such amounts that when brought to a pH reading of 6.8 by addition of sodium hydroxide, the resulting concentration of the sodium salt would be approximately 0.2 per cent. The exact percentage concentration could not be calculated from the molecular weights as most of the organic acids here used are weak acids and their salts are partially hydrolyzed. Even though the pH value is at or near 6.8, it is not to be assumed that complete change of the

acid to its normal sodium salt has occurred. The actual amount of normal sodium salt present is somewhat less than that found by calculation but for the present purpose exact calculation based upon the dissociation constant is unnecessary.

Fifty representative cultures of the colon-aerogenes group, including strains from as many different sources as possible, were selected for the tests. The typical fecal *Bact. coli*, methyl red positive and Voges-Proskauer negative, was represented by 22 strains of either human or animal fecal origin. The *Bact. aerogenes* cultures, methyl red negative and Voges-Proskauer positive, were isolated from soil samples, water and grains, with the exception of a few fecal aerogenes cultures which were also included in the tests. A third set included 3 strains which upon repeated tests gave either a negative or a weakly positive Voges-Proskauer reaction. The methyl red test was consistently negative. The occasional weak Voges-Proskauer reaction, together with the general characteristics of the cultures, indicated a relationship with the aerogenes type. Another group consisted of 3 strains which were consistently methyl red negative—Voges-Proskauer negative. Aside from this the deportment of these cultures also showed a close similarity to the typical aerogenes type.

The different media were filled into test tubes, 8 to 10 cc. per tube and sterilized in the autoclave at 15 pounds pressure for fifteen minutes. All cultures were inoculated from young agar slants by transferring a small amount of growth on the tip of the straight wire. Incubation was at 30°C. The luxuriance of growth was recorded at definite intervals, usually one, two, and four and seven days after inoculation, although where negative results were secured this was frequently extended to thirty days.

A summary of the experiments with different organic acids supplied as the source of carbon is presented in table 2. The results are given here as recorded after seven days incubation since it is unnecessary to present the detailed observations made in each experiment. The several media used as controls are placed first. Here it is seen, as previously pointed out, that in the presence of an available source of carbon such as glucose or lactose,

TABLE 2

Development of the colon-aerogenes group with the organic acid salts as the only source of carbon

	BACT. COLI.		BACT. AEROGENES		RESEMBLING BACT. AEROGENES	
	Methyl red, + Voges-Proskauer, 0		Methyl red, 0 Voges-Proskauer, +		MR. 0 VP. 0 or weak +	MR. 0 VP. 0
	Fecal origin		Soil, water, grains	Fecal		
	Human	Animal				
NUMBER OF CULTURES IN EACH GROUP EMPLOYED IN TESTS.....	12	10	18	4	3	3
Control media:						
Glucose, 0.2 per cent.....	+++ (10) ++ (2)	+++ (5) ++ (4) + (1)	+++ (18)	+++ (4)	+++ (3)	+++ (3)
Lactose, 0.2 per cent.....	+++ (5) ++ (6) + (2)	+++ (2) ++ (6) + (2)	+++ (18)	+++ (4)	+++ (2) ++ (1)	+++ (3)
No source of carbon.....	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.
Organic acid media:						
Sodium salt of, -Acetic.....	+++ (9) ++ (3)	+++ (3) ++ (7)	+++ (7) ++ (8) + neg. (1)	+++ (3) ++ (1)	+++ (1) ++ (2)	+++ (3)

Propionic.....	++ + neg.	(2) (7) (3)	++ +	(5) (5)	all neg.	all neg.	+++ neg.	(1) (2)	all neg.
n-Butyric.....	? or + neg.	(5) (7)	+ neg.	(3) (7)	all neg.	all neg.	+++ neg.	(1) (2)	all neg.
n-Valeric.....	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.	+++ neg.	(1) (2)	all neg.
Iso-Valeric.....	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.	+++ neg.	(1) (2)	all neg.
n-Caproic.....	all neg.	all neg.	all neg.	all neg.	all neg.	all neg.	+++ neg.	(1) (2)	all neg.
Succinic.....	+++	(12)	+++ ++ +	(7) (3) (1)	+++ ++ +	+++ ++ +	+++ ++ +	(3) (3) (1)	+++ +++ +++
Malic.....	+++ ++	(11) (1)	+++ ++	(10)	+++ ++	+++ ++	+++ ++	(3) (2)	+++ +++
Lactic.....	+++	(12)	+++ ++	(8) (2)	+++ ++	+++ ++	+++ ++	(2) (1)	+++ +++

Increasing luxuriance of growth from slight to heavy turbidity is indicated by the plus signs +, ++, and +++ No evidence of growth is designated "neg." The numerals in brackets following these signs show the number of cultures exhibiting the particular luxuriance of growth indicated.

The observations given here are readings taken on the seventh day after inoculation. Incubation at 30°C.

TABLE 2—Continued

	BACT. COLI.		BACT. AEROGENES		RESEMBLING BACT. AEROGENES	
	Methyl red, + Voges-Proskauer, 0		Methyl red, 0 Voges-Proskauer, +			
	Fecal origin		Soil, water, grains		MR. 0 VP. 0 or weak +	
	Human	Animal		Fecal	MR. 0 VP. 0 or weak +	MR. 0 VP. 0
NUMBER OF CULTURES IN EACH GROUP EMPLOYED IN TESTS	12	10	18	4	3	3
Glyceric.....	+++ (11) + (1)	+++ (9) ++ (1)	+++ (16) ++ (2)	+++ (3) ++ (1)	+++ (2) ++ (1)	+++ (2) ++ (1)
Citric.....	all neg.	all neg.	+++ (15) ++ (3)	+++ (4)	+++ (2) ++ (1)	+++ (3)
Tartaric.....	all neg.	all neg.	+++ (6) neg. (12)	+++ (1) neg. (3)	+++ (1) ++ (1) neg. (1)	+++ (2) neg. (1)
Mucic.....	+++ (8) ++ (1) neg. or ? (3)	+++ (8) + or ? (2)	+++ (13) ++ (5)	+++ (3) neg. (1)	+++ (2) ++ (1)	+++ (3)
Malonic.....	+ (8) ? (2) neg. (2)	+ (8) ? (1) neg. (1)	++ (9) + (8) ? (1)	++ (2) + (1) ? (1)	+++ (1) ++ (1) neg. (1)	++ (3)

Oxalic.....	all neg.	all neg.	all neg.	all neg.	all neg.
Benzoic.....	all neg.	all neg.	++ + neg. (13)	(2) (3)	++ + neg. (1)
Salicylic.....	all neg.	all neg.	all neg.	all neg.	all neg.
o-phthalic.....	all neg.	all neg.	all neg.	all neg.	all neg.

all the cultures develop and most of them attain a luxuriant growth. Without the available source of energy no development is apparent.

In the monobasic fatty acid series it was found that the lower acids supported growth while the higher acids were apparently not attacked. The results secured with acetic acid present a decided contrast to those given by valeric and caproic acids, while propionic and butyric acids seem to be intermediate in that they support a moderate or light growth of some of the coli type. All cultures, with one exception, developed in the acetic acid medium and in most cases attained a moderately heavy growth. A retarded development, due evidently to the acetate radical, was apparent. With the usual 0.2 per cent sodium acetate the first visible turbidity usually did not appear until thirty-six to forty-eight hours or more after inoculation and a heavy growth was not attained until the fourth or fifth day. An increase in the amount of sodium acetate from 0.2 to 0.4 per cent caused a correspondingly greater inhibition.

The results secured with propionic acid were especially interesting, for here many of the *Bact. coli* cultures produced a moderate growth while only one strain of *Bact. aerogenes* was able to develop. The coli strains developed very slowly and no turbidity could be discerned until the fourth or fifth day after inoculation. While this acid seemed to offer some promise of affording a means for the separation of the coli and aerogenes types, it was not investigated further at this time since some of the fecal cultures consistently refused to grow while one of the aerogenes type developed readily in every instance. Also, the slow development impaired considerably any usefulness which this acid might possess. It seems probable that the propionate radical exerts a retarding influence as did the acetate. In the presence of n-butyric acid a few of the *Bact. coli* cultures exhibited a meagre development. Whether this scanty growth was due to a limited availability of n-butyric acid or whether the butyrate radical exerted such a marked inhibitory effect as to prevent more than a slight growth was not determined.

Succinic, malic, lactic and glyceric acids were readily utilized by all cultures of both the coli and aerogenes types and usually supported an abundant growth.

Citric acid stands out as especially significant, for here there occurred a distinct separation in the behavior of the different types of the colon-aerogenes group. The *Bact. coli* cultures of fecal origin all refused to develop, while *Bact. aerogenes*, together with the several atypical strains, grew readily and the dense turbidity exhibited by these cultures was in striking contrast to the clear tubes which had been inoculated with the coli cultures. All aerogenes cultures multiplied readily, usually exhibiting turbidity within twenty-four hours and attaining a luxuriant growth by the third or fourth day. Several different samples of citric acid or its salts were employed with similar results.

Tartaric acid supported growth of some of the aerogenes cultures, though not all. *Bact. coli* showed no evidence of growth. Where a utilization of the tartrate was effected by *Bact. aerogenes*, the development was usually rapid and the cultures presented a dense turbidity. Mucic acid, which is quite similar to tartaric acid in structure, supported development of most of the colon strains and all but one of the aerogenes type. The remaining organic acids need little comment. In malonic acid many of the cultures produced a slight to moderate growth, the development of the aerogenes cultures usually being considerably heavier than that of the coli type. Benzoic acid supported the growth of a few of the aerogenes cultures, while oxalic, salicylic and orthophthalic acids were not utilized in any instance.

In all of the synthetic organic acid media, growth of both the coli and aerogenes types appeared first as a light uniform turbidity which gradually became denser and after several days partially settled to the bottom of the tube. Upon agitation a heavy uniform turbidity usually resulted, although a few of the cultures showed a tendency to clump and exhibited a more or less flaky appearance.

Determinations of the hydrogen-ion concentration were made at frequent intervals in all of the experiments. Utilization of the acids was accompanied by a decrease in the hydrogen-ion concen-

tration. In those cases where the organic acids were readily utilized and a luxuriant growth resulted, the pH of the culture was changed from the original 6.7 to 6.8 to a point usually about 8.4 to 8.8, within seven days. This was true of succinic, malic, citric, tartaric and mucic acids. Lactic acid always supported a heavy growth but the change to an alkaline reaction was never as pronounced, the final pH usually ranging from pH 7.2 to 7.6. Glyceric acid presented somewhat the same features, the aerogenes cultures attaining a pH of 7.0 to 7.4 and the coli cultures 7.4 to 8.4. In malonic acid *Bact. coli* developed only to the extent of a light turbidity and caused very little change in the reaction, while on the other hand some of the aerogenes cultures caused a decrease in the hydrogen-ion concentration to pH 8.2 to 8.8. It is possible that traces of impurities in the malonic acid may have been responsible for the slight growth of the colon strains. In every instance where development of the culture was not apparent the hydrogen-ion concentration remained unchanged. In young cultures a slight turbidity could be discerned before a change in the hydrogen-ion concentration could be detected by the colorimetric method.

A comparison of the structural formulae of the organic acids is of some interest.

Acetic	$\text{CH}_3 \cdot \text{COOH}$	Tartaric	$\text{CHOH} \cdot \text{COOH}$ $ \text{CHOH} \cdot \text{COOH}$
Propionic	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{COOH}$		
n-butyric	$\text{CH}_3 \cdot (\text{CH}_2)_1 \cdot \text{COOH}$	Mucic	$\text{CHOH} \cdot \text{COOH}$ $(\text{CHOH})_2$
n-valeric	$\text{CH}_3 \cdot (\text{CH}_2)_2 \cdot \text{COOH}$		$ \text{CHOH} \cdot \text{COOH}$
iso-valeric	$(\text{CH}_3)_2 \text{CH} \cdot \text{CH}_2 \cdot \text{COOH}$		$\text{CH}_2 \cdot \text{COOH}$
n-caproic	$\text{CH}_3 \cdot (\text{CH}_2)_3 \cdot \text{COOH}$	Citric	$\text{C}(\text{OH}) \cdot \text{COOH}$ $ \text{CH}_2 \cdot \text{COOH}$
Lactic	$\text{CH}_3 \cdot \text{CH}(\text{OH}) \cdot \text{COOH}$		$ \text{COOH}$
Glyceric	$\text{CH}_2(\text{OH}) \cdot \text{CH}(\text{OH}) \cdot \text{COOH}$	Oxalic	COOH $ \text{COOH}$
Succinic	$\text{CH}_2 \cdot \text{COOH}$ $ \text{CH}_2 \cdot \text{COOH}$	Malonic	$\text{CH}_2 \begin{matrix} \swarrow \text{COOH} \\ \searrow \text{COOH} \end{matrix}$

Malic	$\text{CH(OH)} \cdot \text{COOH}$ $\text{CH}_2 \cdot \text{COOH}$	Benzoic	$\text{C}_6\text{H}_5 \cdot \text{COOH}$
		Salicylic	$\text{C}_6\text{H}_4(\text{OH}) \cdot \text{COOH}$
		Ortho-phthalic	$\text{C}_6\text{H}_4(\text{COOH})_2$ (1, 2)

Here it is seen that carbon is presented in a number of different combinations, in carboxyl, methyl and alcohol radicals and the benzene ring and with these radicals attached in different ways. The addition of the methyl groups to the carbon chain in the acetic acid series is accompanied by a corresponding decrease in growth. In a number of instances a slight change in the structure of the acid resulted in a decided difference in availability. Compare, for instance, propionic and lactic; oxalic, malonic and succinic; malic and tartaric or tartaric and mucic acids (table 2). On the other hand, several compounds of different structure have presented similar results. The explanation for the failure of *Bact. coli* to utilize citric acid does not seem apparent, since some of the radicals are similar to those contained in several of the acids which were readily utilized. It is possible that the addition of certain linkages to a compound may tie up or prevent the utilization of other carbon-containing radicals which would otherwise be available.

Experiments with citrates

Since it was found that the inability to utilize citric acid afforded a ready means for differentiation of the fecal *Bact. coli* strains from the other members of the colon-aerogenes group, further work was confined to a study of the usefulness of this acid. In addition to the usual citric acid neutralized with sodium hydroxide, several other salts of the acid were employed. Commercial preparations of potassium, ammonium and sodium citrate gave identical results. An experiment with 0.2 per cent sodium citrate (0.277 per cent sodium citrate $\cdot 5\frac{1}{2}\text{H}_2\text{O}$) showing the progressive development of the aerogenes type and the change in hydrogen-ion concentration is given in table 3. As brought out here, the distinction between the two types is usually apparent within twenty-four hours and becomes much more pronounced as the growth of the aerogenes culture's increases in luxuriance. In

TABLE 3

Showing the development of colon-aerogenes cultures in a synthetic medium containing 0.2 per cent sodium citrate as the only source of carbon

DESIGNATION OF CULTURES	LUXURIANCE OF GROWTH AFTER				pH OF MEDIUM AFTER			
	24 hours	48 hours	4 days	7 days	24 hours	48 hours	4 days	7 days
<i>Bact. coli.</i> (fecal)								
Human 1.....	0	0	0	0	6.7	6.7	6.7	6.7
Human 2.....	0	0	0	0	6.8		6.8	6.8
Human 10.....	0	0	0	0	6.8	6.7	6.8	
Human 11.....	0	0	0	0	6.8			6.8
Human 12.....	0	0	0	0	6.8	6.7	6.8	6.8
Human 13.....	0	0	0	0				6.8
Human 14.....	0	0	0	0	6.7		6.8	
Human 15.....	0	0	0	0				6.8
Human 16.....	0	0	0	0			6.8	
Human 17.....	0	0	0	0	6.8		6.8	
Human 18.....	0	0	0	0		6.8	6.8	6.8
Human 19.....	0	0	0	0	6.8	6.8	6.7	6.8
Rat 1.....	0	0	0	0	6.7			6.8
Rat 2.....	0	0	0	0	6.8			6.9
Rat 3.....	0	0	0	0	6.8			6.8
Guinea-pig 1.....	0	0	0	0	6.8			6.8
Guinea-pig 3.....	0	0	0	0	6.8			6.8
Guinea-pig 4a.....	0	0	0	0	6.8			6.8
Rabbit 1.....	0	0	0	0	6.8			6.8
Rabbit 5.....	0	0	0	0	6.7			6.8
Rabbit 8.....	0	0	0	0	6.8			6.8
Sheep 2.....	0	0	0	0	6.8			6.8
<i>Bact. aerogenes</i> (soil, water and grains)								
P ₁	+	++	++	++	6.8	7.0	8.4	8.6
P ₂	++	+++	+++	+++	7.0	7.6	8.8	9.0
2050B.....	++	++	+++	+++	6.9	7.2	8.2	8.6
RCP.....	++	+++	+++	+++	7.1	7.4	9.0	9.0
L.....	++	+++	+++	+++	7.2	7.4	8.0	8.8
2203.....	++	++	+++	+++	7.0	7.4	8.0	8.4
RC-5.....	+	+++	+++	+++	7.2	7.5	8.6	9.0
Va.1B.....	++	+++	+++	+++	7.4	8.2	8.8	9.0
Va.1C.....	+	++	++	+++	6.8	7.2	7.6	8.4
PGF1.....	+	++	++	++	7.0	7.2	7.6	8.4
PGF3.....	+	+++	+++	+++	6.9	7.1	8.0	8.6
PGF4.....	+	+++	+++	+++	6.9	7.4	8.0	8.6
PGF6.....	++	+++	+++	+++	7.2	7.6	8.6	9.0
12755.....	+	++	+++	+++	7.0	7.6	8.8	9.0

TABLE 3—Continued

DESIGNATION OF CULTURES	LUXURIANCE OF GROWTH AFTER				pH OF MEDIUM AFTER			
	24 hours	48 hours	4 days	7 days	24 hours	48 hours	4 days	7 days
<i>Bact. aerogenes</i> (soil, water and grains)—Continued								
79482.....	++	+++	+++	+++	7.0		8.6	9.0
690.....	+	++	+++	+++	6.8	7.0	7.8	8.4
Rice 1.....	+	+++	+++	+++	7.0	7.4	9.0	9.0
Flax 1.....	+	+++	+++	+++	6.9	7.2	8.6	9.0
Fecal aerogenes								
Rabbit 2a.....	+	++	+++	+++	6.9	7.1	7.4	8.4
Rabbit 6.....	++	+++	+++	+++	6.9	7.4	8.6	9.0
Rabbit 9.....	+	++	+++	+++	6.8	7.0	7.8	8.4
Guinea-pig 8B.....	++	+++	+++	+++	7.2	7.6	8.4	9.0
Voges-Proskauer reaction variable, methyl red negative								
PGF 5.....	+	++	++	++	7.0	7.5		8.8
2050 A.....	+++	+++	+++	+++	7.4		8.6	9.0
gp.5.....	0	++	++	+++	6.7	7.0	7.7	8.4

Increasing luxuriance of growth is represented by the plus signs, +, ++ and +++. 0 signifies no visible growth.

this and preceding experiments the temperature of incubation was 30°C. Similar results have been secured at 37°C. With the cultures used in the present investigation either temperature may be employed, although 30°C. is to be recommended for general use as an occasional aerogenes culture may grow with difficulty at body temperature.

Optimum concentration of citrate

In all of the previous work 0.2 per cent citrate was adopted arbitrarily. Since it is well known that varying concentrations of a salt may cause either an inhibition or stimulation of growth it is necessary to obtain definite knowledge of the effect produced by different amounts of citrate upon the behavior of *Bact. aerogenes* and *Bact. coli*. For this purpose the combination of inorganic salts described in the first part of the paper was employed and to this sodium citrate was added in varying concentrations.

Controls which contained no citrate were also employed in every instance. Where the larger amounts—1 and 3 per cent—of citrate were used the hydrogen-ion concentration of the medium was slightly lowered and it was necessary to adjust the reaction to the usual pH 6.7 to 6.8 by means of HCl. For the purpose of

TABLE 4
The effect of various quantities of sodium citrate upon the rate of growth of Bact. aerogenes

CULTURE DESIGNATION	TIME REQUIRED TO SHOW VISIBLE GROWTH IN SODIUM CITRATE CONCENTRATIONS OF							PLAIN BROTH CONTROL
	3 per cent	1 per cent	0.5 per cent	0.1 per cent	0.01 per cent	0.001 per cent	None (control)	
	pH-6.8	pH-6.8	pH-6.8	pH-6.8	pH-6.7	pH-6.7	pH-6.6	
	hours	hours	hours	hours	hours			hours
<i>Bact. aerogenes</i> :								
Rabbit 2a.....	48	19	14	13	12	neg.*	neg.	5
Rabbit 9.....	24	12	9	9	9	neg.	neg.	6
G. P. 8b.....	24	10	10	9	9½	neg.	neg.	6
P 1.....	22	18	14	13	11	neg.	neg.	6
RCP.....	16	9½	10	9½	10	neg.	neg.	5
RC 5.....	24	12	10½	11	12	neg.	neg.	5
Va 1B.....	22	10	10	8	11	neg.	neg.	4½
PGF6.....	20	16	11	10	11	neg.	neg.	6
79482.....	22	12	11½	10	11	neg.	neg.	6
690.....	18	11	11	9½	9	neg.	neg.	5
Flax 1.....	22	10	9½	9	10	neg.	neg.	7
Average time for aerogenes.....	23.8	12.68	10.95	10.09	10.5			5.59
<i>Bact. coli</i> (controls):								
Human 1.....	neg.*	neg.	neg.	neg.	neg.	neg.	neg.	neg.
Human 10.....	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
Human 18.....	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

* Tubes remaining clear at expiration of 4 days were recorded as negative.

determining the comparative rapidity of development in citrate media of various concentrations the procedure of Holm and Sherman (1921) was followed. This consists simply in using the time required by the organisms for production of visible turbidity as a measure of the rate of growth. To insure a uniform inoculation, the inorganic salt-citrate media were tubed in 10 cc. quan-

titles and all tubes in a series representing the range of citrate tested were inoculated with one small loopful, 1 mm. loop, of a twenty-four-hour culture grown in either 0.1 citrate or 0.1 per cent glucose synthetic medium. Eleven strains of *Bact. aerogenes* and three of *Bact. coli* were used. All cultures were incubated at 30°C. and observations made at frequent intervals to note the first appearance of visible growth. The results of this experiment are given in table 4.

It is evident that the higher concentrations of citrate markedly retard the growth of *Bact. aerogenes*. This is especially noticeable in the 3 per cent concentration where the average time required for the aerogenes cultures to produce a visible turbidity was 23.8 hours. The inhibitive effect of citrate has been noted by Holm and Sherman (1921) who found that 0.2 molar sodium citrate in a one per cent pepton solution retarded the growth of *Bact. coli*. The experiment given in Table 4 shows that the inhibitive effect is decreased as the amount of sodium citrate is reduced until at a concentration of 0.1 per cent the most rapid growth occurs. In the 0.01 per cent citrate medium a slightly longer average time is required to show visible growth, though this difference is almost negligible. The 0.001 per cent citrate and the control tubes without citrate failed to show visible growth. Evidently the 0.001 per cent sodium citrate is insufficient to support the continued multiplication necessary to produce a visible cloudiness. To compare the development in plain broth with that in the citrate medium a series of tubes of beef extract pepton broth was included. Here the growth was much more rapid than that in any of the inorganic salt-citrate media.

While the above experiment shows that the highest rate of growth takes place in the lower concentrations of citrate (0.1 and 0.01 per cent) since the least time is required to show visible turbidity, it does not give an idea of the luxuriance of growth attained in the various amounts of citrate after the first visible turbidity has appeared. Thus, although growth was slow in appearing in the 1 and 3 per cent concentrations, ultimately a much more luxuriant development was attained here than in the smaller amounts of citrate. The cultures in 0.01 per cent never showed

more than a light growth while in the 0.1 per cent citrate medium the turbidity was not quite as dense as that exhibited by the 0.5 per cent concentration. Evidently, then, the smaller amounts of citrate are insufficient to support the aerogenes cultures to the limit of their ability to develop. Therefore, to secure the best results in separating the coli and aerogenes types we must choose

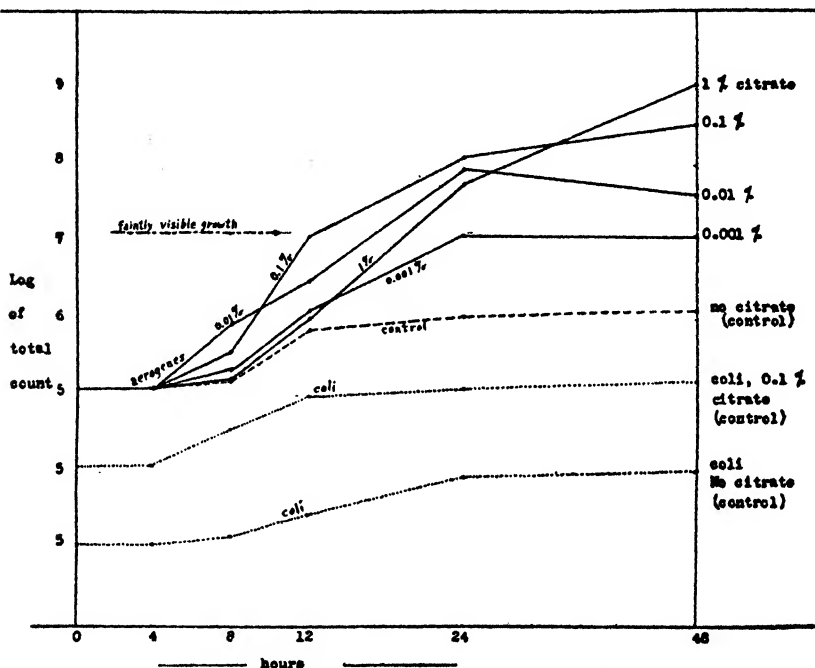


CHART 1

Bact. aerogenes cultures in citrate media of various concentrations are indicated by solid lines (—), without citrate by dash line (---). The *Bact. coli* cultures used as controls are indicated by dotted lines (.....).

a concentration of sodium citrate small enough to permit a fairly rapid development of *Bact. aerogenes* and at the same time one which will supply sufficient available carbon to allow the organism to attain a luxuriant growth.

To show this more clearly additional experiments were performed in which plate counts were made at different time inter-

vals. The medium employed was the same as that previously used and was put up in flasks containing 50 cc. each to which the various amounts of sodium citrate were added. Several sets of flasks, each representing a series of varying citrate concentrations, were inoculated with different cultures of *Bact. aerogenes* and *Bact. coli*. The results of a representative experiment are shown in chart 1. Here we have a confirmation of the previous experiments, for within the first twelve hours after inoculation the rate of growth is most rapid in the 0.1 and 0.01 per cent concentrations of sodium citrate and these are the first to exhibit visible growth. It is not until after twenty-four hours that the lower concentrations are surpassed by the 1 per cent citrate which steadily increased in numbers while the others had practically reached the peak of development or had decreased. The total counts obtained at the end of forty-eight hours presented a series of gradation comparable to the density of growth as shown by macroscopic observation.

The slight multiplication of *Bact. aerogenes* in the control flask which contained no citrate and also that of *Bact. coli* in the 0.1 per cent citrate, as well as without the citrate, was somewhat surprising. It is believed that the increase in numbers in the controls may be explained by the presence of impurities in the inorganic salts. The increase in numbers of *Bact. coli* in the 0.1 per cent citrate cannot be accounted for so readily since it is slightly greater than that in the controls and may be explained either by an incomplete utilization of the citrate molecule, insufficient to produce a visible growth, or else, and more probably, by impurities in the sodium citrate as well as in the inorganic salts.

From the foregoing experiments on the influence of various amounts of sodium citrate in an inorganic salt medium upon the rate and luxuriance of growth of *Bact. aerogenes*, it is evident that a concentration of between 0.1 and 0.5 per cent will allow a fairly rapid development and at the same time will supply sufficient carbon to permit the attainment of an abundant growth. In most of the subsequent work 0.2 per cent sodium citrate (0.277 per cent sodium citrate $\cdot 5\frac{1}{2}\text{H}_2\text{O}$) has been employed.

Effect of small inoculations into citrate medium

It is well recognized that many of the more "fastidious" micro-organisms will grow on certain media only when considerable amounts of old growth have been carried over into the new cultures and that when only a few cells are transferred no growth results. In the previous experiments inoculation was usually performed by transferring on a straight platinum wire a small amount of young culture from agar slants. Occasionally this procedure was varied by using the loop to transfer from one liquid citrate medium to another. Since such methods result in carrying over several hundred thousand or several million living cells with also small amounts of nitrogenous matter representing dead cells and perhaps traces of enzymes, it was deemed advisable to subject *Bact. aerogenes* to a more rigorous test of its ability to initiate growth. To accomplish this purpose the amount of inoculum was reduced so that each tube of citrate medium received only a few cells. These small numbers were secured by transferring a small amount of growth from a twenty-four-hour agar slant through several 10 cc. dilution tubes of sterile water. From the final dilution tube one loopful was inoculated into 10 cc. of citrate medium and another loopful was introduced into an agar tube which was immediately plated. By this procedure it was found that the total number of living cells introduced into the 10 cc. tubes of citrate medium varied from 82 to 670, i.e., the numbers in the tubes immediately after inoculation varied from 8 to 67 per cubic centimeter. All cultures were placed at 30°C. and examined from time to time to note the first appearance of visible growth and also the luxuriance of subsequent development.

Twenty strains of *Bact. aerogenes*, mostly of soil and water origin, were tested in this manner and in every instance they all developed readily and eventually produced a dense turbidity. Owing to the extremely small inoculum, the appearance of the first visible growth was delayed. Turbidity never appeared before eighteen hours and usually not before twenty-four to thirty hours, while a few cultures required thirty-six to forty-eight

hours to produce a visible growth. After the appearance of the first visible cloudiness the turbidity rapidly increased and the cultures became as luxuriant as those which had been inoculated in the usual manner. This would seem to indicate clearly that *Bact. aerogenes* finds in the citrate radical a readily available source of carbon and that it experiences no difficulty in utilizing it for its own needs. Also, it serves to check the results secured by the usual method of inoculation.

Permanence of the ability to utilize citrate

Bacteriological literature is replete with accounts of variability evidenced by different organisms. A few workers have even gone so far as to claim that the various biochemical tests, such as fermentation of sugars, etc., are valueless for distinguishing microbic types. Although the bulk of opinion is opposed to this view, it is necessary nevertheless to study the constancy of a particular biochemical property when advocating its use for the separation of types. The question may be raised whether *Bact. aerogenes* cultures ever lose their ability to utilize citrate or whether the typical fecal *Bact. coli* may acquire this power. Several experiments were undertaken to answer these points in so far as possible.

In the first place, to determine the ability of *Bact. aerogenes* to sustain continued multiplication in the citrate medium, 10 representative cultures were selected and each was transferred through 50 successive transplants in a medium consisting of the usual inorganic salts and 0.2 per cent sodium citrate. Transfers were made at twenty-four-hour, or occasionally at forty-eight-hour intervals. Throughout the entire series of transplants no decrease in either the rapidity or luxuriance of the growth was apparent. Secondly, to determine whether colon-aerogenes cultures would exhibit the same behavior in a citrate medium upon several isolated tests at different intervals, all the available stock cultures were tested on several different occasions during the course of a year. The stock cultures were kept on agar slants and transferred directly to the usual medium of inorganic salts

plus 0.2 per cent citrate. Altogether 41 cultures of fecal origin (methyl red positive, Voges Proskauer negative), 26 from soil, water and grains (methyl red negative, Voges Proskauer positive) and 6 atypical strains were subjected to from 3 to 5 different tests. In every case the behavior toward citrate remained constant and no change either toward a loss or gain of this power could be detected. Indeed, several details were noted which tended to show remarkable constancy. In the collection of cultures there were 2 strains which exhibited a delayed fermentation of citrate. Visible turbidity was not apparent usually until the third or fourth day after inoculation, although the cultures attained a luxuriant growth after that. These 2 cultures, in the several tests in various citrate media made at different times during the course of a year, gave on each occasion the same delayed fermentation. From the foregoing experiments it is seen that *Bact. aerogenes* was capable of utilizing citrate as a source of carbon throughout a continued period of multiplication and that no gain or loss of the power to attack citrate could be found in a series of 73 colon-aerogenes group cultures.

DISCUSSION

From the foregoing experiments it is seen that many of the organic acids offer an available source of carbon for the colon-aerogenes group and in the presence of suitable nitrogen and certain inorganic salts these acids are broken down with resultant development of the organisms. Evidently the organic acids are utilized both as a source of energy and as a source of carbon for construction of the bacterial cell.

The differentiation of the coli and aerogenes types on the basis of citric acid utilization is of special interest. It is well known that these types may be separated on the basis of glucose metabolism (Rogers and associates, 1914 and 1915; Clark and Lubs, 1915; Levine, 1916). Also, the writer (Koser, 1918) has previously shown that a differentiation may be made on the basis of ability to derive nitrogen from uric acid. The present investigation shows that, in addition to the foregoing methods, these types

can be separated by their ability to utilize certain salts of citric acid. *Bact. aerogenes* and several aerogenes-like strains were able to derive carbon from sodium, potassium and ammonium citrate, while the coli type of fecal origin did not possess this power. The luxuriant growth exhibited by the aerogenes cultures in a citrate medium was in direct contrast to the negative results secured with *Bact. coli*. It is noteworthy that *Bact. aerogenes*, which is considered on the basis of certain cultural properties and extensive sugar fermentations to be the most primitive type of the group (Winslow, Kligler and Rothberg, 1919), is able to derive nitrogen and carbon from certain sources which are not available for *Bact. coli*. This seems to constitute further evidence of the primitiveness of the aerogenes type, if we may assume as Winslow and his coworkers have done, that the course of evolution has been marked by progressive loss of fermentative power.

In regard to the question of the value of citrate differentiation in practical water analysis, the writer believes that this must be left open for the present, since there is not a uniformity of opinion in regard to the value of the commonly used methyl red and Voges-Proskauer tests in determining the sanitary quality of water. To determine the usefulness of a synthetic citrate medium for separation of fecal and non-fecal types of the colon group in routine water analysis several additional lines of work must be completed. A larger series of cultures than that employed in the present investigation should be collected from fecal and non-fecal sources and their behavior in the citrate medium studied. In addition, the relative incidence of the citrate utilizing and non-utilizing types in waters of different sanitary quality must be determined.

Aside from any possible applications in sanitary water analysis, the present investigation shows that the organic acids are entitled to a wider use by bacteriologists than that accorded them in the past. Many of the recent studies in bacterial nutrition have emphasized the utilization of nitrogenous bodies, such as amino acids, purines, ammonium salts, etc., and considerable information concerning the fundamental nitrogenous require-

ments of various groups of microorganisms has been brought out. It is apparent that studies of the utilization of carbon-containing compounds, such as the organic acids, should also prove exceedingly valuable in arriving at an accurate knowledge of the nutritive needs of bacteria and that the organic acids offer a valuable series of test substances for the differentiation and classification of bacterial groups.

SUMMARY

The utilization of various organic acids by the colon-aerogenes group was studied. The organic acids were employed as sodium salts and constituted the only sources of carbon in a simple medium composed of inorganic salts including a suitable source of nitrogen. The utilization of the salts of the various organic acids was shown by development of the cultures and by the production of an alkaline reaction.

A number of the salts, such as those of acetic, succinic, malic, lactic, mucic and glyceric acids supported an abundant growth of the colon-aerogenes cultures. Others, as n-valeric, iso-valeric, n-caproic, oxalic, salicylic and ortho-phthalic gave negative results. Several organic acids supported the growth of some but not all of the colon-aerogenes group. Propionic acid permitted a slight or moderate growth of many of the fecal *Bact. coli* strains while the *Bact. aerogenes* cultures, with one exception, were negative. Tartaric acid was readily utilized by a few of the *Bact. aerogenes* strains while the remainder of this type, as well as *Bact. coli*, gave no evidence of growth. Benzoic acid supported a light growth of a few of the aerogenes type.

The most striking results were obtained with citric acid. Here the fecal *Bact. coli* type consistently failed to develop while the *Bact. aerogenes* cultures multiplied readily and produced a luxuriant growth. The hydrogen-ion concentration of the aerogenes cultures decreased from an initial pH 6.7 to 6.8 to 8.4 to 9.0 while that of the coli cultures was unchanged.

Further work with the salts of citric acid showed that identical results could be secured with either potassium, ammonium or sodium citrate.

The citrate, while supplying available carbon for the aerogenes cultures, exerts a retarding effect upon growth. This becomes more pronounced as the amount of citrate is increased. A concentration of 0.1 to 0.5 per cent sodium citrate was found to be most useful for securing growth by *Bact. aerogenes*. Smaller amounts do not supply sufficient available carbon to permit an abundant growth while larger amounts retard the development.

A small inoculation resulting in the introduction of 8 to 67 living cells per cubic centimeter of the citrate medium was sufficient for *Bact. aerogenes* to initiate development.

Ten *Bact. aerogenes* strains were transferred through fifty consecutive cultures in an inorganic-salt citrate medium. No diminution of growth was apparent. Further experiments showed that the ability to utilize citrate is not readily gained or lost and is evidently a stable character.

The organic acids constitute a set of test substances which should be very useful in the differentiation and classification of various groups of bacteria.

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PHYSIOLOGICAL STUDY OF AZOTOBACTER CHROOCOCCUM, BEIJERINCKII AND VINELANDII TYPES

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INTRODUCTION

This investigation deals with the relation of hydrogen ion concentration in specially prepared culture solution, to the growth of three types of *Azotobacter*, namely, *Chroococcum*, *Beijerinckii*, and *Vinelandii*.

A description of the organisms used, as well as some special technic developed by the authors, is noted in detail.

METHOD OF PROCEDURE

1. Determination of hydrogen ion concentration

The electrometric method which was described by Itano¹ and later modified slightly by the same author, was used throughout the investigation. The method was modified so that direct titration as well as direct reading of pH was made possible.

The modifications were as follows:

- a. The Hildebrand electrode was used in place of Hasselbach's.
- b. The Kohlrausch bridge was used for the meter bridge.
- c. The sliding contact rheostat of 100 ohms resistance was injected in the battery circuit and adjusted at the start so that the current from the battery against the normal element balanced at 700 on the bridge wire. In other words, the apparatus was set so that 1.0189 volt against the battery read at 700 on the

¹ Itano, A. Bulletin 167, Mass. Agri. Exper. Station, Amherst, Mass.

bridge. On this basis the following circular disc with a pointer was constructed so that pH can be read directly from the bridge reading (graph I). This modification eliminates much calculation. It is, however, necessary to use the former method wherever such factors as gas tension, temperature and barometric corrections are to be considered.

Although the colorimetric method has been used very often by other investigators, the authors, realizing its inadequacy, especially in connection with the pigmenting organisms, used the electrometric method described above. It was also found that the "salt error" plays an important rôle in such a synthetic medium as Ashby solution.

II. Culture medium and adjustment of its hydrogen ion concentration

Ashby solution of the following composition was used:

Mannitol.....	20.0 grams
KH ₂ PO ₄	0.2 gram
MgSO ₄ ·7H ₂ O.....	0.2 gram
NaCl.....	0.2 gram
CaSO ₄ ·2H ₂ O.....	0.1 gram
H ₂ O.....	1000 cc.

CaCO₃ was not added except in case of comparative study as control.

The pH was determined by titrating electrometrically with N/10 HCl and N/10 NaOH as in the results noted below:

To prepare the media of different pH, the following procedure was adopted:

1. Ashby solution (–CaCO₃) of double strength was prepared.
2. To this, the determined amount of H₂O, N/10 HCl or N/10 NaOH and 10 cc. of inoculum was added so that the total volume became equal as noted in table 1.

III. Organisms used in the investigation

Three types of *Azotobacter* of the description given in table 2 were employed.

These organisms were isolated by the author's method² which was found to be very effective in obtaining pure *Azotobacter* free from *B. radiobacter*.

About 1 gram of soil was placed in 20 cc. Ashby solution, in a 150 cc. Erlenmeyer flask and incubated about three days. (The length of incubation varies for different types.) As soon as the medium became turbid, a loopful of the culture was

TABLE 1
Final preparation of the media of different pH

DESIRED pH	NUMBER OF					TOTAL VOLUME	FOUND pH
	Double strength medium	Inoculation	N/10 HCl	N/10 NaOH	Sterile distilled water		
	cc.	cc.	cc.	cc.	cc.	cc.	
Control*	90	10			100.0	200	7.0
4.5	90	10	0.2		99.8	200	4.0
5.0	90	10			100.0	200	5.0
5.5	90	10		0.5	99.5	200	6.0
6.0	90	10		1.0	99.0	200	6.3
6.4	90	10		1.3	98.7	200	6.6
6.6	90	10		1.6	98.4	200	6.7
6.8	90	10		1.7	98.3	200	6.8
7.0	90	10		2.0	98.0	200	7.0
7.5	90	10		2.4	97.6	200	7.4
8.0	90	10		3.0	97.0	200	7.6
8.5	90	10		3.5	96.5	200	8.0
9.0	90	10		4.2	95.8	200	8.3
10.0	90	10		5.8	94.2	200	9.2
11.0	90	10		12.0	88.0	200	10.3

* Contains CaCO₃ while all others, none.

plated out and the colonies were fished out and examined for *Azotobacter* in Meissner solution as usually employed. Further the growth of the organism in Ashby solution and the nitrogen fixation were tested. The organism thus obtained was acclimatized just before the inoculation.

The distribution of these organisms, in relation to the reaction of the soils examined, is noted in table 3.

² This method was perfected by Yamagata in collaboration with K. Aoi.

TABLE 2
Description of organisms

	NAME OF ORGANISMS		
	Chroococcum type	Beijerinckii type	Vinelandii type
Form and size	Short plump rod, gen. in pairs. 2.3x3.4	Large oval. 2.3x4.7	Oval. 1.8 x 3.4
Motility	Motile, in young culture only	No	Motile
Coloration on Ashby agar (28 to 30°C.)	A. White B. Brownish black	White Sulfur-yellow	White Greenish yellow: soluble
Coloration in Ashby solution (28 to 30° C.	A. White, turbid B. Brownish black surface film	White, turbid White sediment, pellicle formation	Greenish yellow Yellowish pink
Colony on Ashby agar plate (28 to 30°C.)	Round, pasty, dark concentric ring in the center	Somewhat round moist, wrinkled surface	Round, semi-transparent
Nitrogen fixation per gram mannit	6.96 mgm.	5.58 mgm.	9.12 mgm.

A, young culture; B, old culture.

TABLE 3
Distribution of *Azotobacter*

REACTION OF SOIL	NUMBER OF SOIL SAMPLES EXAMINED	NUMBER OF SOILS CONTAINED AZOTOBACTER		
		<i>Azotobacter chroococcum</i> type	<i>Azotobacter Beijerinckii</i> type	<i>Azotobacter vinelandii</i> type
Acid.....	119	0	0	0
Neutral.....	76	3	11	0
Slightly alkaline.....	62	26	20	0
Alkaline.....	43	37	0	3
Sum	300*	66	31	3

* These samples represent various cultivated soils in Japan, covering the country from the extreme north to the south.

The table indicates the distribution of *Azotobacter* relative to the reaction of the soil. *Azotobacter chroococcum* is most widely distributed and in the alkaline soils; *Azotobacter Beijerinckii*, is less common and in nearly neutral soils; and *Azotobacter vinelandii*, least common and in the alkaline soils.

Again, the geographical distribution of these organisms, especially of *chroococcum*, is worthy of note. They are found generally in warm climates where naturally the amount of humus is

TABLE 4
Growth of Azotobacter chroococcum type

NUMBER OF FLASK	ORIGINAL pH	RATE OF GROWTH			FINAL pH, 96 HOURS	NUMBER OF BACTERIA IN 20 CC. MEDIUM† 96 HOURS INOCULUM, 190,000 (NUMBER IN THOUSANDS)
		48 hours	96 hours	240 hours		
Control*	7.0	+	+	+	7.2	484,000
1	4.0	—	—	—	4.0	190
2	5.0	—	—	—	5.5	210
3	6.0	—	—	—	5.8	10,000
4	6.3	—	±	+	6.0	80,000
5	6.6	±	+	+	6.2	108,000
6	6.7	±	+	+	6.3	116,000
7	6.8	+	+	+	6.3	120,000
8	7.0	+	+	+	6.4	128,000
9	7.4	+	+	+	6.7	136,000
10	7.6	+	+	+	6.8	148,000
11	8.0	+	+	+	6.9	176,000
12	8.3	+	+	+	6.9	156,000
13	9.2	+	+	+	6.9	148,000
14	10.3	+	+	+	8.0	140,000

—, no growth; ± doubtful growth; +, positive growth.

* Contains CaCO_3 while all other flasks contained solution without it as has been stated previously.

† Breed Direct Counting method was used. Breed, R. S., *Centbl. Bakt. Abt.* II, 1911, vol. 30, 337-340.

almost lacking and the soil is neutral or alkaline, and very few in cold climates except where wood ash or CaCO_3 is applied, or where sea shell deposits are found. That means, in general, that the acid condition exists where the humus is present in abundance and is lacking in the alkaline salts, thus preventing the growth of *Azotobacter*, and it is especially true with *A. chroococcum* on account of its association with alkali.

INOCULATION OF MEDIA

One cubic centimeter of forty-eight hour old culture in Ashby solution ($-\text{CaCO}_3$ —reaction adjusted to pH 7.0 with N/10 NaOH) was transferred into 19 cc. of the culture solution of respective pH so that the total volume became 20 cc. in every case.

TABLE 5
Growth of Azotobacter Beijerinckii type

NUMBER OF FLASK	ORIGINAL pH	RATE OF GROWTH			FINAL pH, 96 HOURS	NUMBER OF BACTERIA IN 20 CC. MEDIUM 96 HOURS INOCULUM, 122,000 (NUMBERS IN THOUSANDS)
		48 hours	96 hours	240 hours		
Control	7.0	+	+	+	7.1	164,000
1	4.0	—	—	—	4.1	122
2	5.0	—	—	—	5.2	200
3	6.0	—	—	—	5.8	5,600
4	6.3	—	+	+	5.9	15,600
5	6.6	—	+	+	6.3	20,800
6	6.7	—	+	+	6.5	45,000
7	6.8	—	+	+	6.5	62,200
8	7.0	+	+	+	6.5	69,000
9	7.4	—	+	+	6.8	50,000
10	7.6	—	+	+	6.8	45,200
11	8.0	—	+	+	7.0	18,200
12	8.3	—	—	+	7.5	8,000
13	9.2	—	—	+	7.8	3,000
14	10.3	—	—	±	8.4	400

RESULTS

The results are noted in table 4.

Table 4 indicates that the reaction of the media was changed more or less toward acid during the growth of the organism. The greater change took place on the alkaline side. It is also apparent that better growth took place in media of pH 7.0. A marked beneficial effect of CaCO_3 on growth is indicated by the fact that in the control, Ashby solution with CaCO_3 , the organism grew to 484 millions while the maximum growth was 176 millions in the others. It is interesting to note that pH 7.0 has a slight inhibitory effect. The optimum pH seems to lie be-

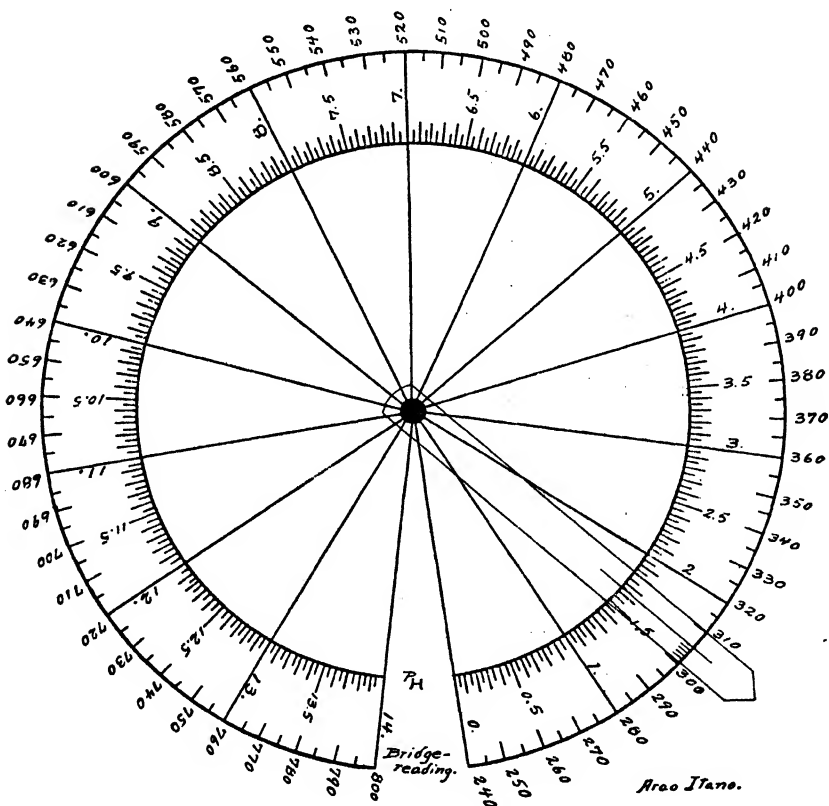
tween 7.45 to 7.60, which was calculated by the authors adopting an arbitrary method as indicated below:

$$\frac{8.0 + 6.9}{2} = 7.45$$

$$\frac{8.3 + 6.9}{2} = 7.60$$

Graph 1.

T_H Reader.



where 6.9 represents pH where the inhibitory action was manifested somewhat markedly; 8.0, where the maximum growth took place; and 8.3, where the decrease in growth was indicated.

Table 5 indicates that the rate of growth is more markedly influenced at different pH values than is the case with the other organisms. For instance, at pH 7.0 good growth of *A. Beijerinckii* took place at the end of forty-eight hours while at all other reactions the organisms were inhibited. The inhibitory influence seems to increase with variations above or below pH 7.0. Only slight difference exists between the control and flask 8 where the pH is optimum. This seems to indicate that this organism is not influenced by the presence of CaCO₃ or at least not so much

TABLE 6
Growth of Azotobacter vinelandii type

NUMBER OF FLASK	ORIGINAL pH	RATE OF GROWTH			FINAL pH, 96 HOURS	NUMBER OF BACTERIA IN 20 CC. MEDIUM 96 HOURS INOCULUM, 180,000 (NUMBERS IN THOUSANDS)
		48 hours	96 hours	280 hours		
Control	7.0	+	+	+	7.0	944,000
1	4.0	—	—	—	4.0	160
2	5.0	—	—	—	5.3	300
3	6.0	—	—	+	5.9	500
4	6.3	—	±	+	6.0	1,200
5	6.6	—	±	+	6.3	10,800
6	6.7	—	±	+	6.3	17,600
7	6.8	—	±	+	6.5	21,400
8	7.0	±	+	+	6.5	22,200
9	7.4	+	+	+	6.8	28,000
10	7.6	+	+	+	6.9	36,000
11	8.0	+	+	+	7.0	72,000
12	8.3	+	+	+	7.1	64,000
13	9.2	+	+	+	7.3	60,000

as the other strains. The optimum pH seems to lie between pH 6.65 to 6.75 which was obtained according to the arbitrary methods as follows:

$$\frac{7.0 + 6.5}{2} = 6.75$$

$$\frac{6.8 + 6.5}{2} = 6.65$$

Table 6 indicates that the behavior of *A. vinelandii* is similar to that of *Azotobacter chroococcum* except the inhibitory action

by pH below 7 was slightly greater. Much greater beneficial effect of CaCO_3 is noted, viz., 944 millions in the control and 72 millions in flask no.11. The optimum pH seems to lie between pH 7.5-7.7 which was determined as follows:

$$\frac{8.0 + 7.0}{2} = 7.50$$

$$\frac{8.3 + 7.1}{2} = 7.70$$

SUMMARY AND CONCLUSIONS

1. The culture solution used in this investigation, Ashby solution without CaCO_3 , served the purpose very well.

TABLE 7

AZOTOBACTER	pH	
	Optimum	Limiting
<i>Chroococcum</i>	7.45 to 7.60	5.80
<i>Beijerinckii</i>	6.65 to 6.75	5.80
<i>Vinelandii</i>	7.50 to 7.70	5.90

2. By preparing media of different pH as described, the amount of food and concentration of other ions were kept uniform.

3. Itano's modified method made the titration easy.

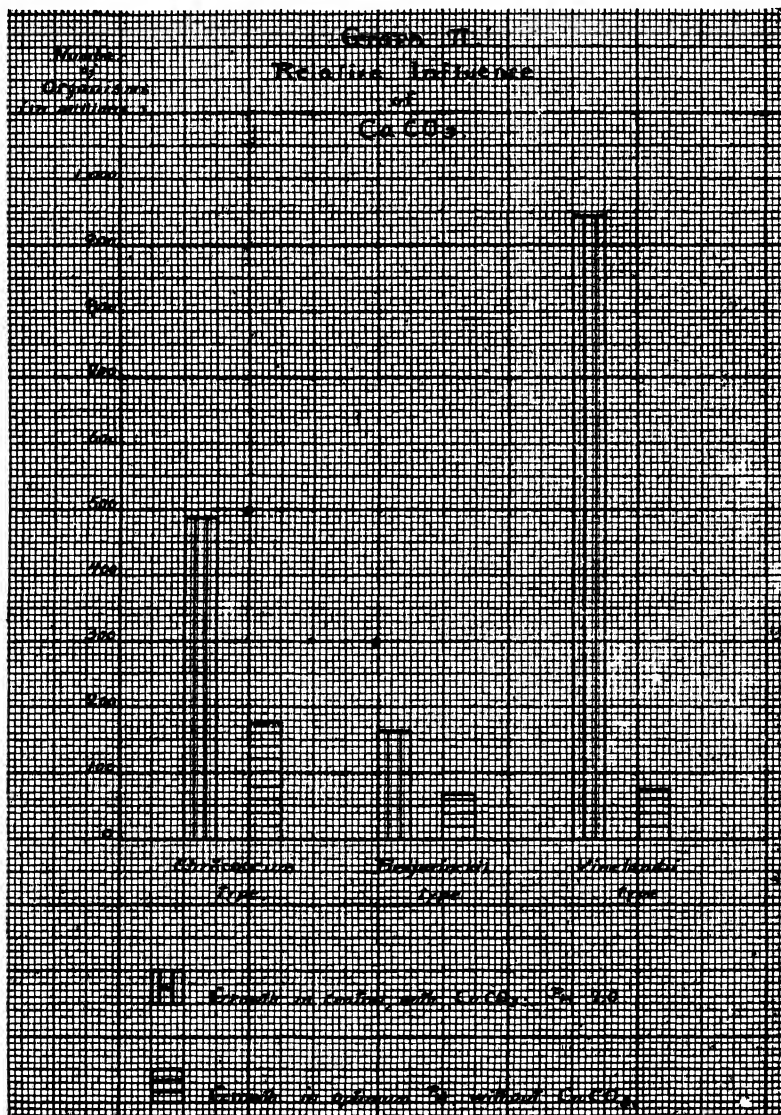
4. A pure culture of *Azotobacter*, free from *B. radiobacter* was obtained from soil very easily by using the Yamagata-Aoi method.

5. The distribution of *Azotobacter* in the cultivated soil in Japan was comparatively restricted; only 33 per cent of the total number of samples examined contained the organisms. This figure is much lower than those given by various foreign investigators. This may be due to the fact that a large percentage of soils in Japan are acid.

6. The optimum pH and limiting pH < 7.0 are found in table 7.

7. As noted in tables 5, 6, and 7, the growth of the organisms, at different pH, and after various periods seem to vary greatly.

That is, at the end of forty-eight hours, the growth of *A. chroococcum* took place at all pH's above 6.6; *A. Beijerinckii*, only in pH 7.0; *A. vinelandii*, at all pH's above 7.0.



8. The influence of calcium carbonate on *Azotobacter* seems to vary with the species. The *Beijerinckii* type grew very well in the absence of CaCO_3 while the other two types, especially *vinelandii*, are very sensitive. This is shown graphically in graph II.

9. At the end of ninety-six hours, the following different degrees of toleration of hydrogen ion concentration, by each organism, were observed: viz., *A. chroococcum* grew distinctly at all pH's above 6.6; *A. Beijerinckii*, from pH 6.3 to pH 8.0; *A. vinelandii*, at all pH's above 7.0. At the end of two hundred and forty hours, *A. chroococcum* and *A. vinelandii* behaved similarly, or both organisms grew distinctly in all pH's above 6.0 while *A. Beijerinckii* grew from pH 6.3 to pH 9.2. Further investigation to determine the influence of OH ions on *Azotobacter*, in presence of CaCO_3 is in progress.

A preliminary investigation on some phase of this experiment was carried out by the colorimetric method under Prof. J. K. Wilson of Cornell University, to whom the authors are indebted.

ANTIGENIC RELATIONSHIPS OF BACT. TYPHOSUM

I. AGGLUTINATION¹

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The essential identity of antigenic structure of strains of *Bact. typhosum* is frequently brought into question by reports of "atypical" strains of typhoid bacilli and also by the reported success of attempts to divide this group of organisms into so-called "types" or "groups." Opposed to the latter conception is the generally accepted opinion that most, if not all, strains of *Bact. typhosum* are much alike and present marked cultural and antigenic uniformity.

The present work was prompted by the isolation from the urine, blood, or feces of several patients, some of whom were thought to have typhoid fever, of Gram-negative bacilli which, because of their source and cultural characteristics, apparently belonged to the typhoid-paratyphoid group. These strains failed to agglutinate with, or absorb agglutinin from, the laboratory sera. On being tested with sera prepared from two recently isolated typhoid strains some of the cultures were identified as true typhoid organisms. This observation suggested a study of the antigenic relationships of a large number of strains of *Bact. typhosum*; particularly as similar investigations had previously led to discordant conclusions and in some instances were based on scanty material.

The problem, presented, then, was to determine whether or not typhoid strains are alike or dissimilar in antigenic structure, and, if not alike, whether the differences are qualitative or merely quantitative.

¹ Dissertation presented to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

For convenience, only the results of agglutination tests will be presented in this paper, since they form an entity which may be considered and discussed apart from the more satisfactory and conclusive results obtained with the complement-fixation reaction.

The numerous reviews (Arkwright, 1914; 1921: Buchanan, 1919: Tulloch, 1915), previously published, of the factors affecting agglutination make still another summary of the literature superfluous; and references to the findings of other workers will be limited to those which have a direct bearing on the experimental data presented below.

EXPERIMENTAL DATA

For the study of the antigenic relationships of *Bact. typhosum* approximately 150 typhoid cultures were collected from various laboratories situated in all parts of this country, in Canada, and in the Canal Zone. This series may be considered representative of *Bact. typhosum* as found in North America.

It may be noted here that all strains have been identified in this laboratory as *Bact. typhosum* by their cultural, agglutination, and complement-fixation reactions, and in the case of strains from other laboratories, their identity has been ascertained by someone other than the writer. In no case has a strain been included as *Bact. typhosum* which gave reactions incompatible with its classification as such.

The simplest and most obvious method of determining possible antigenic differences is agglutination. Although the results cannot be accepted as final, owing to the occurrence of relatively or absolutely inagglutinable strains, still it was hoped that agglutination tests would serve to point out some differences between the strains and provide a clue to those strains which should be more carefully and thoroughly studied.

The agglutination experiments can be divided into two parts: Those done with living saline suspensions and those with formalinized broth cultures prepared by Dreyer's method.

I. Agglutination tests with saline suspensions

For these experiments the organisms were grown on extract agar slants for twenty-four hours, and washed off with sufficient sterile physiological saline solution to give suspensions of appropriate and approximately equal density. Serum dilutions were made in glass bottles and 0.5 cc. quantities distributed from these stock dilutions to small tubes, to which 0.5 cc. quantities of the suspensions were then added. The tests were incubated for two hours in the 37°C. water-bath and then placed in the ice-box. Readings were made the following morning. In this way all the available cultures were tested, lots of 10 to 40 strains being done at one time.

It is unnecessary to give in detail the results obtained with all strains, for a single protocol will provide the salient features. From the examination of such a protocol (table 1; sera EU, EW, Rawlings) it is obvious that some strains are agglutinated more strongly than others when tested with any one serum; that the readily and poorly agglutinable strains, as determined by their reaction with one serum, do not necessarily coincide with a similar classification based on the results with another serum; and that some strains are not agglutinated in significant dilutions by any of the sera used. For example, strain W was agglutinated in dilutions of 1:3200, 1:800, and 1:400 by sera EU, EW, and Rawlings, respectively; strain U in dilutions of 1:400, 1:400, and 1:1600; and strain A in dilutions of 1:400, 1:800, and 1:400. Strain S was not agglutinated by any of the three sera in dilutions as low as 1:100. With serum EU, then, these strains would arrange themselves $W > U$ and $A > S$, in order of agglutinability; with serum EW, W and $A > U > S$; and with serum Rawlings, $U > W$ and $A > S$. Entirely similar phenomena were found in the other experiments.

That these preliminary findings could not be unconditionally accepted as demonstrating antigenic variation was shown in an experiment in which a number of Rawlings strains were tested. The Rawlings strain, according to Nichols (1915), was isolated in 1900, in England, from the spleen of a fatal case of typhoid

TABLE 1
Agglutination tests with saline suspensions

STRAIN	SERA			
	EU	EW	Rawlings	V
A	400	800	400	
C	400	400	200	
D	400	200	400	
E	200	200	400	
F	200	400	200	
G	400	400	-100	
H	400	400	100	
K	200	200	100	
L	400	400	400	
M	800	400	100	
N	800	400	800	
O	800	800	800	
P	800	800	800	
R	100	-100	400	
S	-100	-100	-100	400
T	800	800	1600	
U	400	400	1600	
V	100	100	200	
W	3200	800	400	
X	-100	100	-100	800

The figures gives the highest dilution in which complete agglutination occurred. A figure preceded by a minus sign (-) signifies that complete agglutination was not present in this and higher dilutions.

TABLE 2
Agglutination tests with formalinized suspensions

ANTIGENS	SERA								
	FK	AV	R	Rawlings	DW	EW	CH	EU	V
R	114	90	100	100	25	40	45	20	88
V	114	80	90	100	100	20	33	40	100
AV		20							
CH	128	110	60	75	50	60	100		25
DW	114	70	30	75	100	80	88	40	
ET	128	110	60	100	125	60	76	40	100
EW	128	100	100	25	100	100	112	80	112

The figures represent agglutination in percentage of the titre of the serum for its homologous strain.

Where no figure is given, complete agglutination did not occur in any dilution tested.

fever. A transplant of this culture was received by the Army Medical School at Washington in 1908, and has since been widely distributed in this country. In response to our request for

TABLE 3
Agglutination tests with Rawlings and Hopkins strains

STRAIN	SERA									
	EU	EW	Rawlings	FK	Sm	La	Ro	Ba	Lo	Li
D	800	800	400	400	-20	-20	-20	-20	-20	640
L	800	400	800	1600	-20	80	80	-20	160	1280
M	-100	-100	-100	3200						1280
Y	400	-100	-100	800	-20	-20	-20	-20	160	160
AB	400	400	400	3200	20	40	80	-20	160	1280
AC	200	400	200	3200	20	-20	80	80	160	640
AE	-100	-100	400	-200	-20	40	-40	20	160	Spont.*
AN	400	800	400	800	-20	-20	-20	-20	-20	-40
AR	800	400	800	6400	-20	80	-20	20	-20	1280
BC	400	400	800	-200	20	40	-20	160	-20	Spont.
BY	800	400	-100	1600	-20	160	-20	-20	-20	-40
CD	400	400	400	6400	-20	160	160		160	1280
CS	800	800	400	1600	-20	-20	80	-20	160	640
CW	800	800	400	6400	-20	-20	40	20	160	640
DN	200	400	800	3200	-20	-20	80	160	160	1280
EA	400	1600	200	1600	20	-20	80	160	160	1280
ET	400	800	800		-20	160	-20	160	160	Spont.
N	800	1600	800		-20	-20	-20	40	80	
AA	400	800	800		-20	-20	-20	40	20	
AD	1600	800	400		-20	-20	80	20	-20	
AF	1600	3200	800		40	20	-20	-20	40	
AL	1600	1600	800		20	-20	20	-20	80	
AY	1600	800	800		-20	-20	-20	20	-20	
BF	200	200	800		-20	-20	-20	160	-20	
CN	-100	100	-100		-20	-20	20	20	-20	
ES	800	800	400		-20	-20	-20	40	-20	

Rawlings strains: first 17 (D to ET).

Hopkins strains: last 9 (N to ES).

Monovalent rabbit sera: EU, EW, Rawl., FK.

Human sera (inoculated): Sm, La, Ro.

Human sera (patients): Ba, Lo, Li.

Figures are dilution in which complete agglutination occurred; i.e., 800 = complete agglutination in serum diluted 1:800.

Figures preceded by minus sign indicate incomplete or no agglutination in this and higher dilutions.

*Spont., Spontaneous agglutination.

typhoid cultures we received seventeen Rawlings strains from sixteen different laboratories. These were tested for agglutinability with the three sera used in the previous experiments and also with two sera from typhoid patients and three from prophylactically inoculated normal persons (table 3). At the same time a number of cultures of the Hopkins strain were similarly examined.

Recently, twenty months after the original experiments, the Rawlings strains have been again tested with two sera (table 3, Sera FK and Li). One of these is a rabbit serum prepared with a recently isolated strain (FK) and the other was obtained from a patient. Differences similar in character to those found in the earlier experiments are still noticeable in the later ones, in spite of the fact that for over a year and a half the environment of all the cultures has been the same.

II. Agglutination tests with formalinized broth suspensions

The marked differences in agglutinability noted in the preceding series of tests can not be accepted as conclusive evidence of variation, in view of the numerous sources of error inherent in the agglutination method and of the variable reactions disclosed by the several Rawlings strains. Although, then, these findings are not valid evidence of *definite* antigenic difference, they may be accepted as evidence of *possible* antigenic difference, and it is from this standpoint that they are considered to possess some value.

A number of strains which apparently differed more or less widely from each other in the experiments described above were selected and formalinized broth suspensions were made of them after they had been transplanted in infusion broth daily for two weeks (Dreyer, 1919). Nineteen such suspensions were made, each from a different strain.

The tests were carried out in the same way as the preceding series, except that incubation was done at 55°C. and that a change was made in the method of diluting the serum. Instead of using dilutions which proceeded by geometrical progression, the dilu-

tions progressed arithmetically, the interval between successive dilutions representing about 10 per cent of the titer of the serum for its homologous strain. This procedure possesses two advantages: First, the higher dilutions lie closer together, which tends to bring out slight differences between strains which are strongly agglutinated; and second, results with different sera can be readily compared by charting agglutination in terms of the percentage of the serum's titer rather than as serum dilutions.

In a few experiments all nineteen strains were tested, but later, as similarities appeared, this number was reduced to seven. Several more tests were conducted with these suspensions before it was decided to abandon the use of agglutination tests for the solution of the problem.

TABLE 4

Diagnostic agglutination tests with Rawlings and Hopkins strains

	POSITIVE		NEGATIVE	
	Rawlings	Hopkins	Rawlings	Hopkins
Ba.....	5	4	10	5
Lo.....	11	3	5	6
Li.....	12		2	

The reactions (table 2) obtained by this technic differ only slightly in detail, and not at all in character, from those obtained with the saline suspensions. With most of the sera several strains were agglutinated to titer; although, the particular strains which so react, vary with the serum used. Similarly, all strains (except AV) were strongly agglutinated by one or more sera. This observation is of importance, since it proves that failure of a suspension to be agglutinated was not due to inherent inagglutinability.

DISCUSSION

In the experimental work reported above, a number of typhoid strains have been tested for agglutinability with a number of monovalent anti-typhoid rabbit sera and with several human sera. The strains employed have differed in source, age, and environment since isolation. A preliminary series of experiments

was performed in which saline suspensions of the organisms were tested, in groups of 10 to 40 strains, for agglutinability with three sera. After all the available cultures had been thus examined, nineteen strains were selected as representative of the collection. These were used in the preparation of formalinized broth suspensions by Dreyer's method, and then tested for agglutinability.

The outstanding feature of all the experiments is the failure of the strains to react alike. While nearly all the strains examined were well agglutinated by one or another serum, yet it is apparent that no one monovalent serum would serve to identify them all as *Bact. typhosum*. In other words, the failure of a strain to agglutinate with a given serum must in most instances be attributed to lack of appropriate agglutinin in the serum rather than to inherent inagglutinability of the bacteria. This is particularly true if formalinized broth suspensions are employed, for all but one of these were agglutinated to full titer by more than one serum.

Inagglutinability, either absolute or relative, of typhoid cultures has been attributed to various factors. Among these are lack of motility, insufficient transplantation since isolation, recent contact with immune serum, tendency to spontaneous sedimentation and agglutination, contamination by the bacteriophage of d'Herelle, or the use of serum of the wrong antigenic group. In the present investigation all but the last named can be disregarded since the cultures used have been shown to be agglutinable, as was indicated in the preceding paragraph.

The one question left for discussion, then, is the possible presence of antigenic groups or at least of antigenic differences among typhoid strains. References may be cited either in favor of, or against, the presence of such groups. Dreyer, Gardner and Walker (1921) maintain that such differences as exist in the agglutinability of typhoid strains are merely quantitative, so that the relative agglutinability of any two or more strains may be expressed as a numerical ratio or "coefficient of agglutinability." Opposed to this view is the theory of antigenic groups as put forward by Weiss (1917), by Downs (1921), and by Hooker (1916-1917). Of these the first two have used agglutination and

agglutinin-absorption tests and the last has employed the complement-fixation reaction. Weiss found 7 and Downs 4 groups, while Hooker was content with only 3. This question will be left for fuller discussion in another paper, but it is apparent that the experimental results reported here do not support either view. Dreyer's opinion is flatly contradicted, and to this extent the proponents of the "group" theory are upheld. But, on the other hand, there is no conspicuous evidence of clear-cut antigenic types. From the standpoint of the agglutination reaction, the relationships of the different strains are so intricately complex that the possibility of classification appears remote. Even though it could be accomplished, it would have to be based on very fine and arbitrary distinctions.

The most interesting phase of the work is the series of tests conducted with the Rawlings strains. All of the strains have been tested with at least nine sera. Some of the tests were performed shortly after the cultures were received, the others after the lapse of twenty months, during which time all strains were maintained in the same environment. We must assume that the cultures sent to us as Rawlings strains are really descendants of the original strain and have not been erroneously labelled as such. Taking it for granted that our assumption is correct, the evidence is definite and clear-cut that changes have taken place, and that Rawlings strain in laboratory "A" is not identical in its reaction to agglutinating sera with the Rawlings strain in laboratory "B."

It is felt that the differences brought out in these experiments may serve to explain some of the discrepancies noted when Widal reactions are reported from two or more laboratories. In table 4 are tabulated the results obtained when the sera of different patients were tested against the seventeen Rawlings and Hopkins strains. Complete agglutination in a dilution of 1:40 is regarded as a positive reaction, and no agglutination (at 1:40) as negative. The results with the serum last tested show more uniformity but even this serum failed to give complete agglutination with two of the strains, despite the fact that its titer was above 1:1280. The presence of such marked differences in cultures derived from supposedly the same source is proof of the fallibility of a method of grouping based upon the agglutination reaction.

CONCLUSION

1. Agglutination tests of a number of typhoid strains show that such strains present considerable antigenic differences in so far as such tests furnish valid evidence.

2. These differences are not sufficiently clear-cut and definite to permit the designation of types or groups; particularly since the descendants of a single culture may show similar variations.

3. The failure of a strain to be agglutinated by an anti-typhoid serum is not proof of the non-typhoid nature of the strain; nor is failure of a serum to agglutinate a strain of *Bact. typhosum* proof of a lack of typhoid agglutinin in the serum.

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THE RELATION OF DIGESTIVE ENZYMES AND FERMENTS TO THE PHENOMENON OF d'HERELLE

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Since the French edition of "The Bacteriophage" by d'Herelle was published in October, 1921, and the English edition in March, 1922, it hardly seems necessary to preface this discussion with the customary explanatory remarks concerning the phenomenon of bacteriophagy.

It will be recalled that in d'Herelle's discussion on the nature of the bacteriophage, three hypotheses are submitted. First, that "the bacteriophage is derived from the superior organism in its reaction to the bacterial invasion by the production of a principle which provokes the destruction of the bacterium." Two possibilities, he says, are admissible under this hypothesis. (1) That the principle mentioned is of a diastatic nature. (2) That the principle is particulate, an organic being, capable of developing outside of the organism at the expense of the bacteria. Second, that "the bacteriophage may be derived from the lysed bacterium itself." According to Kabeshima (1920a and b) the bacteria secrete a diastase with autolytic functions. Bordet and Cuica (1920, 1921, 1922) have sponsored a combination of these first two hypotheses as a probable explanation. Third, that "the bacteriophage is an autonomous organism, an ultramicrobe parasitizing the bacteria." The latter view is that held by d'Herelle.

We have concerned ourselves in this work with a study of various digestive enzymes and ferments with the idea of determining what, if any, relation exists between these substances and

the phenomenon of bacteriophagy. Davison (1922) in a recent article concludes that the lytic principle in d'Herelle's phenomenon is possibly an enzyme. Working with one and five per cent solutions of trypsin in normal saline and twenty per cent alcohol he showed that there was no bacteriolytic activity of these solutions against cultures of normal dysentery bacilli, although his solutions were chemically active. Combiesco (1922) working with enterokinase and trypsin, treating each with alcohol for periods varying from twenty-four to seventy-two hours and heating at 55°C. and from 60 to 70°C., reports the presence of a transmissible lytic principle except where the higher temperatures were used. He failed to demonstrate any lytic activity with papain after 40 passages. He states that the activity found in enterokinase is much stronger than that found in trypsin. Pico (1922) claims to have demonstrated the lytic principle in solutions of pancreatin, trypsin, papain and papayotin (carase, papain or papase), after heating to 100°C. Bachman and Aquino (1922) have reported the presence of a lytic principle in pancreatin. Kuttner (1923) reports the presence of a lytic principle in 25 and 50 per cent glycerol extractions of the small intestines of guinea pigs and acetone extractions of the liver tissue. We have work now in progress which bids fair to confirm these findings.

Desiring to cover the field as fully as possible it was necessary to go to the open market and obtain enzymes prepared for biologic purposes. In each case we have included the dealer or manufacturer's name and such data regarding the preparation as might be of interest.

In testing the bacteriolytic activity of Diastase of malt (Eimer and Amend), 10 per cent solutions in water having pH values of 6.9 and 7.4 respectively were used. With each enzyme tested the optimum pH for chemical activity as well as for bacteriolysis was used. In this experiment 1 per cent pepton water (pH 8.0) bacterial suspensions were prepared by adding one drop of a twenty-four hour broth culture of the organism to each tube and these were then incubated for twelve hours. The enzymes were placed in solution and the hydrogen ion concentration determined, after which these solutions were filtered through a Mand-

ler No. 4 candle, incubated and cultured to prove their sterility. To 8 tubes containing twelve hour bacterial suspensions of Shiga and Flexner dysentery bacilli (4 tubes in each set), were added amounts of a 10 per cent water solution of the enzyme varying from 0.5 to 1 cc. To the same number of tubes of bacterial suspension were added similar amounts of the enzyme solution of the higher pH concentration. Control bacterial suspensions and medium were of course included in the series, the whole being placed in the incubator at 37.5°C. Chemical activity of the enzyme solutions was then determined with the material remaining and both solutions were found to be markedly active chemically when tested with starch. Readings were taken at periods of twelve hours up to seventy-two hours. No macroscopic bacteriolytic activity was evident during this period of observation. Upon plate subcultures there were found to be no "sensitive" colonies after twenty hour hours nor were we able to demonstrate any "colonies" of bacteriophages upon agar slant subcultures from the various tubes. These results were uniformly obtained after repeated passages.

In testing the bacteriolytic activity of Taka-Diastase (Parke, Davis & Co.), claimed by them to liquefy 300 times its weight of starch in ten minutes, sample 2526314, a 10 per cent solution in 0.85 per cent saline of pH 7.5 was used. Bacterial suspensions of Shiga dysentery bacilli were prepared as in the preceding experiment. The enzyme solution was filtered, incubated and tested for sterility as described above. The test was then applied in a like manner. The material was markedly active chemically when tested with starch solution. Readings taken at twelve hour intervals during incubation up to seventy-two hours showed no evidence of bacteriolytic activity. There were no "sensitive" colonies or colonies of bacteriophages demonstrated in subculture. A known bacteriolysant from our stock used as a control gave the characteristic findings.

In the study of Pepsin, two different commercial preparations were used. Parke, Davis & Co. powdered pepsin, U.S.P. marked 1:3000, sample 2550561 and Eli Lilly & Co. granular pepsin, U.S.P. The difficulty attending the study of this enzyme in

connection with this problem is manifestly one of reaction. For this reason the enzyme was tested under a variety of conditions, varying reaction, strength of enzyme in solution, subsequent dilutions with bacterial suspensions and medium employed. Both preparations of pepsin were used in five and ten per cent water solutions. Bacterial suspensions of normal Shiga dysentery bacilli were prepared and tested as in the preceding experiments. The hydrogen ion concentration of the pepsin solutions was varied from pH 6.4 to 7.4. In some cases sodium hydroxide was used to neutralize the acid present (in which case the chemical activity of the pepsin was destroyed) and in other samples we made use of calcium carbonate followed by weak alkali such as sodium acetate or milk of lime which was added very cautiously. Solutions of pepsin made neutral or slightly alkaline in this manner do not lose their chemical activity and can be reactivated upon the addition of small amounts of hydrochloric acid. Untreated samples of pepsin in solution, pepsin solutions to which as much as 0.3 per cent hydrochloric acid were added and samples which were first neutralized as described above and then made slightly acid gave macroscopic lysis in the tubes of bacterial suspensions. Upon plate and agar subcultures however, there were found to be no "sensitive" colonies or colonies of bacteriophages. The time in which lysis took place varied directly with the acidity of the solutions. We are inclined to the view that where "clearing" took place, it was more a question of reaction and addition of inhibitory substances (acid) than of lytic activity of the pepsin. Indeed a subsequent study of the effect of different normalities of hydrochloric acid and sodium hydroxide when 0.5 cc. amounts were added to bacterial suspensions of Shiga dysentery bacilli showed that $N/5$ hydrochloric acid would cause "clearing" in the tubes after twenty-four hours incubation while the $N/5$ sodium hydroxide would not clear all the tubes in the series until forty-eight hours had passed. Similarly $N/10$ hydrochloric acid caused "clearing" in all the tubes in seventy-two hours while sodium hydroxide of this concentration had no effect when added in the same quantity.

Davison stated that N/5 sodium hydroxide would check the action of a bacteriophage; that N/1 sodium hydroxide killed dysentery bacilli and also lysed them; that 20 per cent hydrochloric acid killed the cultures but did not lyse them. We found as did he, that the organisms treated with hydrochloric acid stained with more difficulty.

It is apparent then that bacterial suspensions of Shiga dysentery are more sensitive to hydrochloric acid than to sodium hydroxide and so for this added reason we feel that the "clearing" of the bacterial suspensions with addition of acid pepsin solutions was due to the acid present and not to the presence of any lytic activity of the enzyme.

To further substantiate this view the effect of acid pepsin and alkaline pepsin on living and dead (killed by heating to 80°C. for twenty minutes) bacterial suspensions of both Shiga and Flexner dysentery bacilli was tested by making bacterial counts. Stained smears from cultures were prepared both before and after incubation with the enzyme solutions to determine if the organisms were lysed. Subcultures were made before and after incubation to determine the presence of living organisms. These were found to be negative after incubation. The bacterial counts of the Flexner and Shiga dysentery suspensions at the beginning of the experiment (twelve hour cultures) were 20,400,000 and 14,320,000 respectively. After twenty-four hours incubation with the acid pepsin solution the counts were 9,440,000 and 8,750,000 while after incubation for twenty-four hours with the alkaline pepsin solution the counts were 15,200,000 and 9,540,000 respectively. The stained smears before incubation in each case were graded plus-minus in regard to the amount of debris present. After incubation for twenty-four hours with the pepsin solutions the stained smears were graded two plus in each case. The killed cultures of Flexner and Shiga dysentery at the beginning of the experiment gave counts of 19,200,000 and 15,400,000 respectively. After twenty-four hours incubation with acid pepsin the counts were 4,320,000 and 6,020,000 while after incubation with the alkaline pepsin the counts were 5,440,000 and 8,360,000. The stained smears at the beginning

of the experiment were graded one plus in each case while after incubation with the acid and alkaline pepsin solutions the stained smears were recorded four plus in regard to the amount of debris present. The acid pepsin solution was chemically active while the alkaline pepsin was not.

A commercial preparation of Pancreatin was obtained from Parke, Davis & Co. One per cent solutions of this preparation had a marked chemical activity upon fat and carbohydrates but its tryptic activity was only slight. We have been unable to obtain a satisfactory preparation of trypsin as such, though we believe these results with pancreatin substantiate those of Davison with regard to this particular enzyme. Five and 10 per cent solutions in physiological saline and also in 20 per cent alcohol were tested. The absence of any bacteriolytic activity in any of the solutions was uniformly definite. There were no "sensitive" colonies or colonies of bacteriophages on subcultures after repeated passages. A preparation of Metagen, which is claimed by Parke, Davis & Co. to be a combination of fat soluble A, water soluble B and C vitamins was tested in this same series and found to possess no bacteriolytic qualities nor did it aid the growth of the suspensions when compared with the control suspensions. Pancreatin was tested against bacterial suspensions of Flexner dysentery, *Bact. coli* and *Bact. typhosum* with the same negative results as with Shiga dysentery.

While the digestive function of the pancreas is of much importance and has been well understood for some time through the chemical study of this preparation called pancreatin, investigators have long thought there was also another most important secretion from this gland which until recently could not be isolated. We refer to the preparation known as "insulin" or the active secretion from the Islands of Langerhans of the pancreas. In connection with a study of methods of preparing "insulin," having obtained a very active preparation, 350 mgm. of the powder being capable of reducing the blood sugar in rabbits as low as 0.04 per cent and producing convulsions or death, we thought it would be worth while to test its bacteriolytic activity. Four-tenths of a gram of "insulin" was dissolved in 20 cc. of sterile

physiological salt solution. It was then filtered through a Mandler No. 4 candle and a subculture agar slant made to test its sterility. Bacterial suspensions of *Bact. coli*, *Bact. typhosum*, Parathphoid A and B, Shiga and Flexner dysentery were prepared having a series of 3 tubes for each organism. The solution of "insulin" was then added according to the d'Herelle technique for testing filtrates, i.e., 2 drops in the first tube, 10 drops in the second and 2 cc. in the third. Controls were of course included in the series. It should be stated that this preparation of "insulin" was very slightly acid to litmus. The entire series was placed in the incubator and observations made every twelve hours. There was no evidence of any lytic activity in any of the tubes nor were there any "sensitive" colonies or colonies of bacteriophages upon subsequent subcultures. These results obtained after repeated passages.

A commercial preparation of Papain (Merck, sample 12469-LS 55821), was then tested as heretofore described against twelve hour bacterial suspensions of both Shiga and Flexner dysentery bacilli. This preparation was an old one but yet fairly active chemically. There was a uniform absence of any bacteriolytic activity after repeated passages. We may mention that experiments with the bile salts have also repeatedly given us these same negative results.

SUMMARY

Commercial preparations of various enzymes including diastase of malt, Taka-diastrase, pepsin, pancreatin and papain were tested for bacteriolytic activity against twelve hour bacterial suspensions of Shiga dysentery and in some cases against Flexner dysentery, *Bact. coli* and *Bact. typhosum*. All of these preparations were active chemically. The enzymes were tested under a variety of conditions simulating the optimum conditions for bacteriolysis and chemical activity. In no case, except with pepsin, was there any suggestion of bacteriolytic activity. We believe the action present in solutions of pepsin is due to the acid reaction and not to the activity of the pepsin. A preparation of "insulin" which is active chemically by the biologic test was tested for its

bacteriolytic activity against members of the colon, typhoid and dysentery group of organisms. Solutions of the bile salts and a solution of Metagen (vitamines) have been likewise studied.

CONCLUSIONS

If the lytic principle in d'Herelle's phenomenon is an enzyme, this enzyme is not diastase of malt, Taka-diastase, pepsin, a combination of trypsin, steapsin and amylopsin as found in pancreatin or a combination of a proteolytic and amylolytic enzyme as found in papain. The lytic principle is not "insulin," the active secretion of the Islands of Langerhans of the pancreas. It is not related to the bile salts or to vitamins in so far as this can be determined by commercial preparations which are available. If the diastatic theory as to d'Herelle's lytic principle is to prove tenable more information of a chemical nature must be brought to bear upon the problem.

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AN APPARATUS FOR TUBING SEMI-SOLID MEDIA

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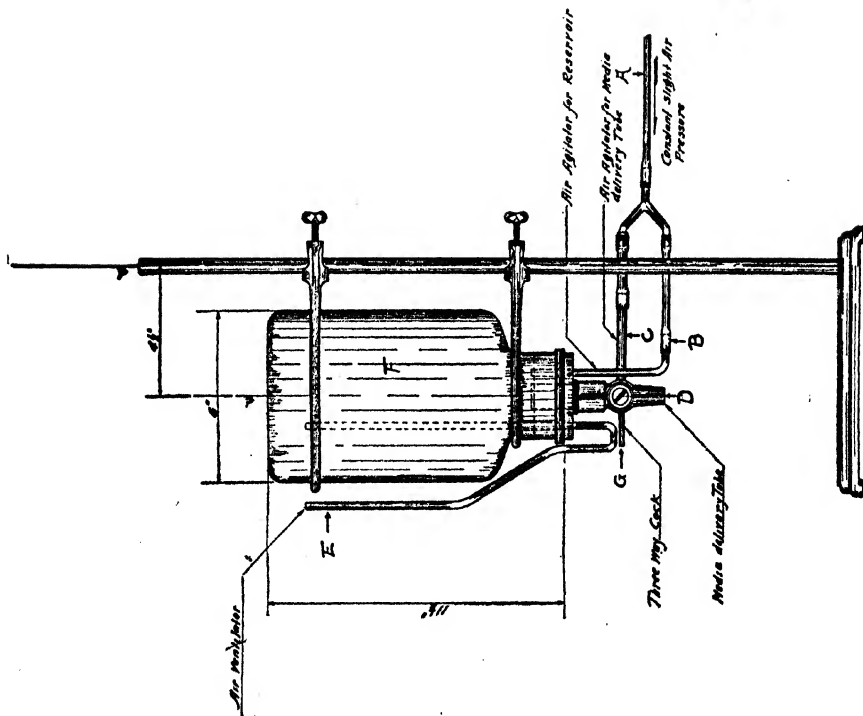
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We have had considerable difficulty in finding a satisfactory method for tubing semi-solid media, such as those containing egg, meat or brain. Upon inquiry of other workers, we find they experience the same difficulties. Methods commonly in use are cumbersome, mussy, and require the use of large tubes. The method presented here enables us to tube egg and meat medium almost as readily as one can tube bouillon. Spilling, and soiling of the mouth of the culture tube, so annoying in other methods, is reduced to a minimum, and even medium sized culture tubes (16 to 17 mm. bore) can be filled very rapidly and neatly. A description and diagram of our apparatus is herewith appended in the hope of assisting others whose work requires the use of large amounts of such media.

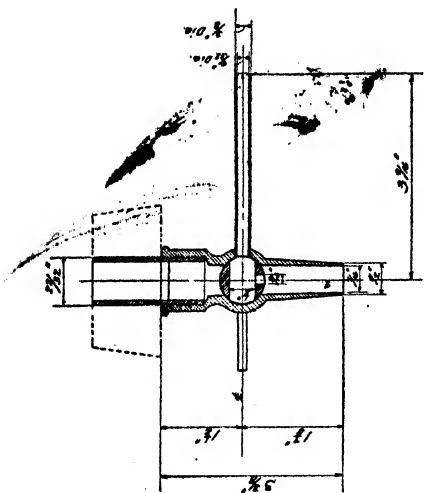
Essentially this apparatus consists of a reservoir, which is a 3-liter, wide-mouthed (65 mm. diameter) glass bottle, a three way stopcock, an inlet and outlet tube for air pressure and a ring stand.

It may be operated either by vacuum or by air pressure. To operate by air pressure, tube *A*, figure 1, is connected to a compressed air line. Tube *B* allows a slow stream of air to enter the reservoir *F* at the bottom which keeps the contents well mixed and enables the operator to get equal proportions of liquid and solid in each tube. Tube *C* is connected to the stopcock for the purpose of removing any solid matter which may become lodged in the stopcock and also for agitation. Tube *E* is the air outlet.

To fill a tube, when using air pressure, the stopcock is opened by raising lever *G* until it points vertically upwards. This cuts off the flow of air through *C*, and allows a free passage of the well



SECTION THROUGH THREE-WAY COCK
Scale Full Size.



APPARATUS FOR FILLING
SEMI-SOLID MEDIA

mixed medium into the culture tube. The stopcock is then closed by lowering the lever to a horizontal position pointing away from the operator which reestablishes a flow of air through *C*. Solid medium is thus dislodged from the upper half of stopcock and again set in agitation. If any solid material should become lodged



FIG. 2

in the lower half of the stopcock, it may readily be discharged by turning the cock so that the lever is pointing horizontally toward the operator. This enables the air coming through tube *C* to pass through the lower half of the stopcock and out at the nozzle *D*.

The photograph, figure 2, shows the appearance of the apparatus when operated by vacuum.

To operate with vacuum, tubes *A*, *B* and *C*, figure 1, are removed entirely. The hole where tube *B* enters the reservoir is stoppered by inserting a glass rod. Tube *E* is connected to a vacuum line. The air enters through tube *C* keeping the stopcock clear and keeping the contents of the reservoir *F* well mixed. The stopcock is manipulated the same as when tubing by air pressure.

THE ATTENUATION OF BACTERIA DUE TO TEMPERATURE SHOCK¹

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I. INTRODUCTION

The phenomenon of attenuation of bacteria is so closely related to that of stimulation that it seems almost impossible to deal with one without making considerable reference to the other. Within certain limits it may be true that a stimulus for one bacterial faculty, may at the same time attenuate the organism in some other manner before it fails as a stimulant. Heat sometimes alters an organism in such a way that one function may be attenuated while others are not. Pasteur, according to Fisher found that heat may cause the Anthrax organism to lose its virulence while at the same time not losing its ability to grow and to multiply.

Before the discussion of attenuation of bacteria is carried further it is important that the term be defined so as to prevent misunderstanding. The word attenuation is derived from *attenuis*, to make thin. As used, attenuation means the lessening of one or more functions of bacteria due to abnormal conditions, temporary or prolonged. A wider use of the term was at first considered, that is, the lessening of all of the functions of an organism. However this wide meaning of the term was found untenable due to the fact that our knowledge of bacteria is largely gained by observations of their separate functions. Attenuation is used as an opposite of the term stimulation in the following discussion and

¹ This is a résumé of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in bacteriology in the Graduate School of the University of Illinois, 1922.

refers to one or more bacterial functions and not to the total of bacterial functions. Thus as far as the use of the term goes, a culture of bacteria may be attenuated as to one function and at the same time be stimulated in other respects.

The subject of the attenuation of bacteria divides itself into three important fields of study: (a) Attenuation of virulence, a field of science which has revolutionized medical practice; (b) attenuation and stimulation of functions of bacteria for commercial advantages, a field of study promising wide application in the manufacturing industries; (c) attenuation of the normal, physiological functions of bacteria, phenomena which are rapidly gaining attention because of their relation to the problems of evolution.

There is no intention here to go into the subject of the attenuation of microorganisms as used in producing immunity in living things but simply to mention how thoroughly grounded it is and of how great service to man it has been. Considering the remarkable results obtained in this field it seems rational to believe that other bacterial functions than that of virulence might be manipulated and certain advantages obtained thereby.

There is more or less material to indicate that some of the functions of microorganisms may be altered for definite periods of time. Takamine, in the manufacture of diastase altered *Aspergillus oryzae*. Adams speaks of the modification of yeast cultures in glycerol manufacture. Wehmer reports the loss of oxalic acid producing power by *Aspergillus niger*. Euler and Svanberg speak of temperature adaptation of yeast. LaFevre and Round suggest that certain organisms may acquire salt tolerance and perhaps later require a certain amount of salt for best development. Zikes reports that he changed the generation time of certain yeasts by prolonged cultivation at abnormally low temperatures.

II. PURPOSE OF THIS WORK

It was the general purpose of the following investigation to gather further data as to the possibility of suppressing the bacterial function of multiplication. While it has been generally considered that the increased keeping quality of pasteurized milk

is due entirely to reduction in numbers of bacteria, still the question may be raised whether a part of this keeping quality might not be due to the attenuation of bacteria during the process of pasteurization. It is the object of the following work to measure if possible any change in the generation time of certain bacteria of market milk before and after the temperature shock of pasteurization.

III. METHODS OF STUDY

Thirty-eight samples of raw market milk were plated on one per cent lactose agar and from these plates were selected 144 predominating colonies. These organisms were then added to litmus milk and incubated for fifteen days at 20°C. From litmus milk cultures there were selected at the end of fifteen days, nine representative organisms taking into consideration their action on litmus milk.

Each of the above nine cultures was then taken separately and comparisons made as to the effects of exposure to a temperature of 145°C. for thirty minutes on its rate of multiplication in sterile milk. Table 1 gives the result of comparisons of the generation times of each organism before and after the temperature shock.

To measure properly any possible attenuation which might occur in bacteria subjected to a heat shock, it is necessary to take into consideration certain variable influences in a milk medium which are more or less unfavorable to bacterial development. It has been the belief of some bacteriologists that milk contains antibodies similar to those in blood.

In order to eliminate the many complex factors involved in the use of fresh milk as a medium for the growth of organisms in this study, only separated milk which had been sterilized in the autoclave for one hour at 15 pounds pressure was used. Large amounts of this milk were sterilized at one time so that at least half of the comparisons of generation time of bacteria reported here were made in different small portions of the same batch of milk, all of which was sterilized at one running of the autoclave. These flasks of sterile milk were allowed to stand at 37°C. for several days to make sure of sterility.

TABLE 1
Effect of temperature shock upon bacterial increase

HOURS	UNTREATED BACTERIA PER CUBIC CENTIMETER	INCREASE	HEAT-TREATED BACTERIA PER CUBIC CENTIMETER	INCREASE
Series 8. <i>B. coli communis</i> No. 222.1113033 held at 20°C.				
0	1,550		518	
8	7,460	4.8	1,470	2.8
16	55,100	7.3	3,940	2.6
24	337,000	6.1	17,800	4.5
32	2,850,000	8.4	69,000	3.8
40	30,700,000	10.7	230,000	3.3
48	Coagulation		1,090,000	4.7
56			4,100,000	3.7
64			16,600,000	4.0
72			67,000,000	4.0
80			221,000,000	3.2
88			Coagulation	
Series 9. <i>Bact. lactis acidii</i> (B) No. 222.2223034 held at 20°C.				
0	26,700		22	
8	280,000	10.4	168	7.6
16	4,190,000	14.9	890	5.2
24	57,100,000	13.6	7,800	8.7
32	586,000,000	10.2	61,000	7.8
40	Coagulation		508,000	8.3
48			3,200,000	6.2
56			32,500,000	10.1
64			257,000,000	7.9
72			Coagulation	
Series 10. <i>Bact. lactis acidii</i> (B) No. 222.2223034 held at 20°C.				
0	54,000		1,000	
8	716,000	13.2	7,000	7.1
16	6,740,000	9.4	18,900	2.6
24	61,200,000	9.0	91,000	4.8
32	Coagulation		538,000	5.9
40			3,580,000	6.6
48			29,700,000	8.2
56			182,000,000	6.1
64			659,000,000	3.6
72			Coagulation	
Series 11. <i>Micrococcus</i> No. 222.3333623 held at 20°C.				
0	83		7	
2	98	1.18	6	0
4	212	2.16	11	1.83
6	330	1.55	25	2.27
8	502	1.51	55	2.20
10	940	1.87	82	1.49
12	1,130	1.20	137	1.67

To each of the two flasks of sterile milk in series 9 and 10, there was added 1 cc. of pure culture from a twenty-four hour growth in sterile milk. Then one flask of each series was left raw and one was pasteurized. These two flasks were then brought to the same temperature and agar plates made at the end of various periods as shown in table 1. One per cent lactose agar was used and colonies were counted at the end of two days at 37°C. and three days at 20°C.

In the case of series 8 and 11, the attempt was made to start each flask of a series at as nearly as possible the same number of bacteria per cubic centimeter. This was done by counting the number of bacteria in the milk culture and then calculating how much it would require of the pure milk culture to leave about equal numbers of bacteria per cubic centimeter after pasteurization of one of the duplicates. In order to arrive at this approximate number of bacteria per cubic centimeter, it was found necessary to determine the pasteurization reduction factor for each organism before starting to work with it.

The method of obtaining plate counts was as follows. Five different dilutions of each sample on 1 per cent lactose agar were made and plated in triplicate giving fifteen plates from which to obtain the bacterial count of the sample. In other words the seventeen determinations of numbers of bacteria showing progressive increase of bacteria in series 8 for example were obtained from 255 plates. The fourteen determinations in series 9 were obtained from 210 plates, and the same rule was carried through the determinations given in the other series. It was considered advisable to make what might be called excessive numbers of plates in order that the factor of variation due to the plate method might be reduced to its lowest point. Test tubes containing 9 cc. of sterile distilled water, were used for making dilutions. Vigorous shaking of dilutions was practiced. Care was taken that the 1 cc. of dilution was thoroughly mixed with the agar on each plate. Incubation of plates was for five days at 20°C. and two days at 37°C.

In determining the generation time the method used by Barber has been followed. If the number of bacteria at the beginning of

TABLE 2
Effect of temperature shock on generation times

UNTREATED BACTERIA				HEAT-TREATED BACTERIA			
No. G.	T.	G. T.	Ave. G. T.	No. G.	T.	G. T.	Ave. G. T.
Data from series 8							
		hrs. min.	hrs. min.			hrs. min.	hrs. min.
2.20	8	3 37	2 54	1.41	8	5 40	4 30
2.84	8	2 48		1.34	8	5 57	
				2.12	8	3 46	
				1.93	8	4 8	
				1.66	8	4 48	
				2.18	8	3 39	
				1.88	8	4 15	
				2.01	8	3 58	
				2.00	8	4	
				1.64	8	4 52	
Data from series 9							
3.31	8	2 24	2 15	2.90	8	2 45	2 47
2.87	8	2 3		2.32	8	3 26	
				3.09	8	2 34	
				2.95	8	2 42	
				3.04	8	2 37	
				2.57	8	3 6	
				3.26	8	2 27	
				2.97	8	2 41	
Data from series 10							
3.65	8	2 11	2 25	2.77	8	2 52	3 36
3.17	8	2 31		1.33	8	6	
3.13	8	2 33		2.20	8	3 37	
				2.47	8	3 13	
				2.66	8	3	
				3.03	8	2 38	
				2.53	8	3 9	
				1.81	8	4 24	
Data from series 11							
0.18	2	11	5 25	0.00	2	0	2 7
1.08	2	1 51		0.83	2	2 8	
0.55	2	3 37		1.13	2	1 45	
0.52	2	3 50		1.10	2	1 48	
0.87	2	2 17		0.49	2	4 4	
0.20	2	10		0.67	2	2 58	

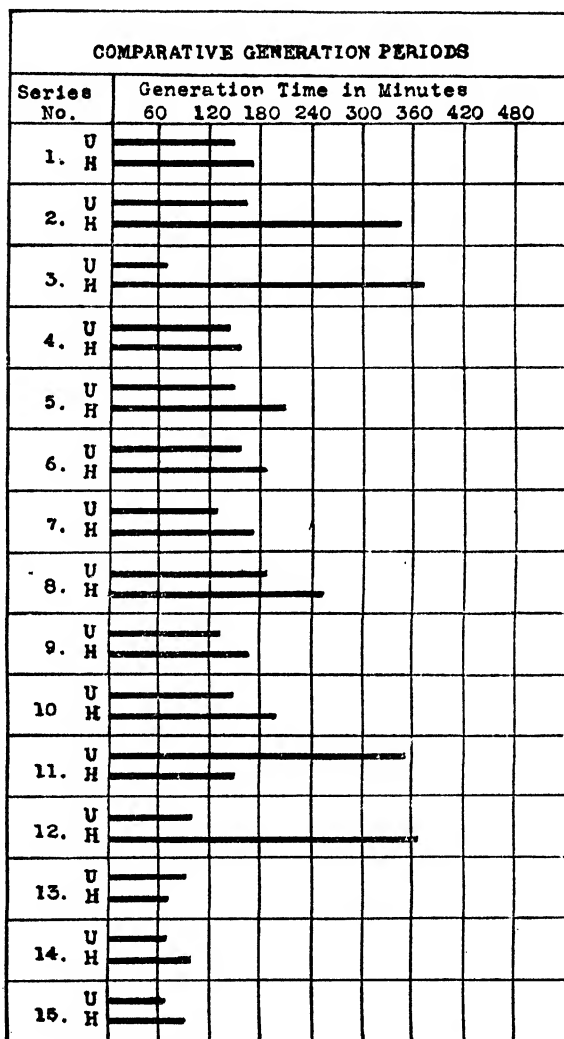


FIG. 1

a period of reproduction (a), and the number at the end of the period (b) be known, then the number of generations (n) can be determined by the formula:

$$2^n = \frac{b}{a}$$

Knowing the number of generations, the generation time can be determined by the following formula:

$$G = \frac{t}{n}$$

The above calculations are based on the assumption that all bacteria in a culture multiply at the same individual rate.

In table 1, the figures under the heading "Increase" were obtained by dividing the number of bacteria per cubic centimeter at the end of an eight hour period by the number at the beginning of the same period of growth. It is only of assistance in aiding the comparisons of rates of increase of bacteria of differently treated cultures. In the graphs showing comparative generation times given in table 1, the letter (U) stands for bacteria untreated by heat and (H) for heat-treated bacteria.

In table 2, "No. G." represents the number of generations which occurred during the period (T) which was eight hours in all the series except 11. The heading (G. T.) stands for generation time in hours and minutes and (Ave. G. T.) represents the average generation time for all the time periods of the series.

DISCUSSION OF RESULTS

Altogether fifteen series of tests were made with fifteen different organisms. Typical results for four of these series have been presented in tables 1 and 2 and the general result of the whole fifteen are graphically presented in the accompanying figure.

In studying the experimental data shown in the graph it is observed that in all the comparative series with the exception of nos. 11 and 13 the average generation time of the bacteria used, has been lengthened by the temperature shock of 145°F. for thirty minutes. Series 11 does not appear to be normal because

of the non-uniformity of the generation time at the beginning and at the end of the growth of the untreated bacteria. It is thought that other factors entered here due to the small number of bacteria per cc., and disturbed the comparisons.

In series 13, 14, and 15 shown on the graph the organisms used were spore bearers and there was no marked difference in the generation times of the untreated and the heat-treated organisms. The heat shock of 145°F. for thirty minutes is considered so far below the temperature exposure which kills the culture that comparisons were of little value in determining attenuation.

In the work with *B. subtilis* spores (series 13) more rapid multiplication was exhibited after than before heat treatment. It would seem that the heat shock of 145°F. for thirty minutes in some way caused stimulation rather than attenuation and it is probable that the temperature exposure is not high enough to threaten in any way the life forces of the spore.

From some of the literature concerning the heat resistance of spores it is evident that this is a very complex subject. H. Weiss reports that the spores of *B. botulinus* are normally destroyed in five hours at 100°C.; forty minutes at 105°C.; and six minutes at 120°C., and that spores one month old are three times as resistant as spores five months old. G. S. Burke found that the spores of *B. botulinus* are more highly resistant to heat when grown in brain cultures than in broth cultures. She reports that exposure to 100°C. or above attenuates the spores to such an extent that the incubation period of the spores is very much lengthened.

It seems very likely that different individual bacteria of a pure culture do not multiply at the same rate although we would expect the average generation time of several large groups of individuals of a pure culture to be quite constant. However, considering that when highly heat resistant bacteria are treated at the pasteurization temperature of 145°F., some individual bacteria die while some individuals are able to withstand even the temperature of boiling water, one would conclude that there is a marked difference between individual bacteria. When a pure culture of milk is treated to a considerable heat shock, the number of bacteria present is always more or less reduced. Those bacteria

which survive differ in some respect from those which die. If this difference were simply one of age of cells it might explain differences in multiplication rates of untreated and heat-treated cultures on the assumption that young and old cells do not multiply at the same rate.

Taking up this subject from another view point, it may be found that there are other characteristics than difference in age of cells which may accidentally accompany ability to survive a heat shock which may have something to do with the generation time of the cell.

To carry further the discussion of possible explanations of the differences in individual bacteria, considering our slight information of the subject would be mere speculation; but there are several additional factors which should not be overlooked. For instance, in two flasks containing pure cultures of the same organism in sterilized milk, when one culture has been subjected to a heat shock and has suffered a reduction in numbers of bacteria per cubic centimeter, the two cultures are no longer comparable. The two cultures differ by the fact that one has considerable numbers of dead cells present while the other culture has none or comparatively few. If we assume that the dead cells are a benefit to the living because of increased vitamine supply, or change in medium, we might conclude that this is a factor tending to shorten the generation time of the surviving bacteria of the culture treated to a heat shock. On the other hand, the rapid cooling of bacteria after a temperature shock is considered by Bushnell to be an attenuating influence in itself. Bushnell says.

The influence of rapid cooling after heating is a matter of considerable importance in the canning industry. The only effect possible that this procedure could have would be that of shock to the bacterial cell. This might possibly devitalize the cell in such a way that it would be more easily destroyed by subsequent heating, or perhaps perish slowly, or not be able to grow under such rather unfavorable conditions as exist in the sealed container.

If it is a fact that the sudden cooling of heated bacteria causes a shock to the bacterial cells, this is an important factor in studying their rate of growth.

Another phenomenon occasionally referred to in bacteriological literature is the influence which numbers of bacteria inoculated into media have upon the growth of the culture.

It is reported by Churchman that when too few organisms are inoculated into certain media there is no growth, while a greater number of organisms produces growth. On the other hand we know that beyond a certain point, numbers of bacteria or their by-products check growth more or less.

Because of the many complex factors involved in growth, it is impossible to say definitely that attenuation is the cause of the different rates of growth of bacteria, heat-treated, and untreated.

VI. CONCLUSIONS

1. Assuming that the 15 organisms here used are representative of the flora of market milk and that these organisms would not relatively act differently in raw milk than in the sterilized milk here used, one seems warranted in drawing the conclusion that the lengthening of the period between production and souring of pasteurized market milk is not entirely due to the reduction in numbers of bacteria but is to some extent due to attenuation of bacteria during pasteurization.

2. Contamination of market milk after pasteurization is from the standpoint of keeping quality more important than the same amount and kind of contamination having survived the process of pasteurization.

3. In pasteurized milk some species of bacteria are killed outright, some are attenuated, and some are stimulated, depending on the resistance of the organism to heat. Those organisms which have a thermal death point below the pasteurizing temperature and time are of course killed off. Those organisms here tested which have a thermal death point a little above the temperature and time of pasteurization are attenuated markedly, while those which have a thermal death point far above the pasteurization time and temperature are attenuated very little or are stimulated to more rapid growth at least for a period.

4. In the development of methods of increasing the commercial life of some preserved food products, it seems very likely that

some benefit can be obtained by emphasizing the attenuation of microorganisms along with their partial destruction.

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THE MECHANICAL BEHAVIOR OF DYES, ESPECIALLY GENTIAN VIOLET, IN BACTERIOLOGICAL MEDIA

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In studying the effects of the use of gentian violet in peptone enrichment media for water borne bacteria, a precipitation of the dye was noted after active growth of the bacteria in the broth. Several possibilities suggest themselves as possible explanations of this behavior. Such precipitation might for example be the result of the formation of a new compound—a decomposition product of the dye, or an absorption product, since no precipitation occurred so long as the broth remained sterile. Kendall and Bly (1922) in studying the changes of the nitrogenous constituents of ordinary media, found that there resulted, upon bacterial growth, an increase in the protein nitrogen fraction of the nitrogen content of the medium. They explained this by stating that an actual production of bacterial protein had taken place. T. B. Robertson (1908) says that the addition of proteins to watery solutions of dyes greatly alters the distribution of dyes between water and lipid, and this alteration is characteristic for each protein. "Hence proteins combine chemically with certain dyes." Kligler (1918) states that the higher the concentration of organic nitrogenous compounds in the medium, the lower the effective concentration of the dye. Churchman (1921) has found that the addition of dead bacterial bodies to gentian violet media will permit organisms to grow which otherwise would not.

That the precipitation noted in the media was not due to reaction with the carbon dioxide or hydrogen, liberated during

bacterial growth, was shown by bubbling CO₂ gas through a broth containing gentian violet, 0.2 per cent lactose and 2 per cent peptone. Experiments were also tried with hydrogen gas both nascent and molecular. In no case was a precipitate obtained. In the presence of washed bacterial growth, molecular hydrogen, bubbled through a homogeneous gentian violet broth, completely decolorized the solution after one and a half hours, and a precipitate settled to the bottom. The hydrogen was not essential, however, as mechanical shaking of a gentian violet solution with washed bacterial protein yielded similar results.

The effect of time, hydrogen ion concentration and the presence or absence of a sugar in the medium, on the precipitation of dye was studied by the following experiment: Two sets of tubes were inoculated with seven cultures. The broth for the first set consisted of gentian violet 1 part to 200,000, 0.2 per cent lactose and 2 per cent peptone. That for the second set differed only in that it contained no sugar. The cultures used were of the colon-aerogenes group—all lactose fermenters. These cultures were incubated at 37°C. As would be expected, gas formed in all tubes containing lactose. In set I (lactose broth), after twenty-four hours only three of the tubes showed a greater amount of precipitation than the corresponding tubes of set II. After forty-eight hours, however, all tubes of the latter set showed a decidedly higher amount of precipitation. After five days incubation, the tubes of set II had become almost colorless, and complete precipitation had taken place, while in set I the maximum decolorization did not amount to over half the dye initially present. The apparent anomaly after only twenty-four hours incubation is to be expected since the presence of sugar would permit a more luxuriant growth during this period.

It took less than twenty-four hours to show the first degree of precipitation. Table 1 gives the values of the hydrogen ion concentration for all the tubes in Sorensen units.

With the exception of culture number 2, the limits of pH for the lactose broth are 4.3 to 4.8, and those for the broth contain-

ing peptone only are 8.0 to 8.5. The dye was taken from alkaline solution, therefore, rather than from the acid solution. In the case of number 2, the peptone broth was distinctly less alkaline than any of the others (pH 7.6) and not all of the dye

TABLE 1

NUMBER	BROTH	pH	PRECIPITATE	APPEARANCE OF LIQUID
2	Lactose	5.0	Light purple	Dark purple
	No lactose	7.6	Dark purple	Faint purple
3	Lactose	4.6	Light purple	Dark purple
	No lactose	8.4	Purplish black	Colorless
4	Lactose	4.65	Light purple	Dark purple
	No lactose	8.0	Purplish black	Colorless
16	Lactose	4.75	Light purple	Dark purple
	No lactose	8.5	Purplish black	Colorless
17	Lactose	4.8	Light purple	Dark purple
	No lactose	8.4	Purplish black	Colorless
19	Lactose	4.3	Light purple	Dark purple
	No lactose	8.3	Purplish black	Colorless
22	Lactose	4.4	Light purple	Dark purple
	No lactose	8.3	Purplish black	Colorless

TABLE 2

BROTH	pH	
	With sterilization	Without sterilization
Peptone	7.2	6.75
Peptone-lactose	6.8	6.5
Peptone-gentian violet	6.8	6.5
Peptone-lactose-gentian violet	6.8	6.5

was removed—though only a small amount remained. In the same instance the lactose broth showed less acidity than the other cultures, pH 5.0 and a little more of the dye had been removed from solution. The removal of dye, then, depends on

the pH of the solution and the above experiments suggest a theory of the effect of dyes on the growth of bacteria which is in line with Loeb's theory of protein behavior. It also leads to a theory of the mechanism of Gram stains, including the limitations of applicability and interpretation, on which work is at present in progress.

In connection with the above experiments, the results obtained were qualitatively confirmed by noting that the addition of small amounts of acid to cultures from which all the dye had been removed, caused it to be reliberated from the precipitate and distributed again throughout the solution, and also that further additions of alkali to this resulting solution again caused all the dye to be precipitated and the solution to become colorless.

Table 2 gives the values of the pH for the various broths used. Where peptone is stated it is as a 2 per cent solution, lactose is 0.2 per cent, and gentian violet 1 part to 200,000.

The pH of washed bacterial precipitate grown in a peptone-lactose broth, centrifuged and washed with distilled water was found to be about 7.6.

DISCUSSION

According to Loeb's theory, a protein is an amphoteric substance which combines with either acids or bases according to whether it is in a solution whose pH is respectively either less or greater than a certain value known as the isoelectric point of the protein in question. On the acid side of the isoelectric point the protein will combine with acids and on the alkaline side it will combine with bases. The isoelectric point of proteins is not necessarily the neutral point, in fact in the case of a large number of proteins whose isoelectric points have been determined all cases except that of gliadin show an acid solution and 75 per cent of those listed are at a pH of 5 or less (1922).

Now gentian violet is a basic dye, and if it combines with protein, this combination should take place on the alkaline side of the isoelectric point, and the amount of combination should increase with increasing alkalinity of solution and decrease with increasing acidity—which was demonstrated to be the case.

If the above theory is true, there should be a point at which the protein no longer takes up a basic dye such as gentian violet but as the acidity is increased it should combine more and more readily with an acid dye such as acid fuchsin. To test this out the following experiment was made: Buffer solutions were prepared between the pH limits of 2 and 5 in steps of about 0.3 of a pH unit. Approximately equal amounts of dead bacterial growth were introduced into tubes, equal volumes of the above buffers added and into each tube a certain quantity of gentian violet was introduced. The tubes were shaken and allowed to stand for several hours. As was expected, the color of the liquid gradually became lighter as the solution became more alkaline so that in the acid solutions very little of the dye was removed while in those solutions of higher pH more and more of the dye combined with the protein. It was found possible to wash out practically all the dye from the protein in the more acid solutions, and less and less of it as the pH increased.

A similar set of tubes was prepared and treated in the same way except that acid dyes were used. In this case the gradation of color after standing went the other way, i.e., more color was taken from the more acid solutions. Both acid fuchsin and ponceau were used and the results were parallel.

SUMMARY

Loeb's theory concerning the amphoteric character of proteins was demonstrated by the fact that the pH was found to regulate to a certain extent the amount of dye taken up by bacterial protein. A point was reached where the bacterial protein no longer took up a basic dye but as the acidity was increased it combined more and more readily with acid dyes. The basic dye used was gentian violet. The acid dyes used were acid fuchsin and ponceau.

Cultures are known to change from Gram-positive to Gram-negative with age. It is possible that this change may be brought about by treating the bacterial protein as growth pro-

ceeds with a solution whose pH is on the side of its isoelectric point opposite to that of the medium at the beginning.

It may also very well be that the effect of certain dyes in selectively inhibiting bacteria may find an explanation in considerations of the above nature.

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A SIMPLE MICROPIPETTE HOLDER

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In the conduct of certain bacterial isolation work in this laboratory, it was found to be advantageous to use a micropipette; and a holder or manipulator was devised that proved very satisfactory. Since its construction was very simple as compared to others that have been described, it was thought that a description of the device would be valuable.

Barber (1914) is generally given credit for originating the micropipette method of isolating organisms and the method is frequently referred to as the Barber method. His apparatus was so complicated that it has not come into general use. Chambers (1923) recently described an improved device but from his description, the mechanism is not much simpler than Barber's original manipulator.

The device here discussed is very much simpler than either of the others, so simple in fact that it was constructed with the simplest tools in half a day. It has been in use for nearly a year and, while it may not have such fine adjustments as the instruments previously described, it has proved entirely satisfactory for bacterial work.

The accompanying illustration shows its principal features. It consists of a rod, *A*, $\frac{1}{8}$ inch in diameter and 10 inches long attached at one end by a ball and socket joint, *B*, to a threaded block, *C*, which moves up and down as the screw, *D*, upon which it works is turned. To its other end is soldered a clamp, *E*, to hold the pipette. It is supported 1 inch from the clamp end by a standard, *F*, through which it passes by means of a right angle reverse bend, in such a manner as to be free to move only as the ball and socket end is raised or lowered. The standard, *F*,

about 2 inches high, is fastened to a Bausch and Lomb Type A mechanical stage, *G*, by removing the slide holder and attaching the standard in its place. The screw, *D*, operating the long end of the rod, is held in a U-shaped piece of brass, *H*, by passing

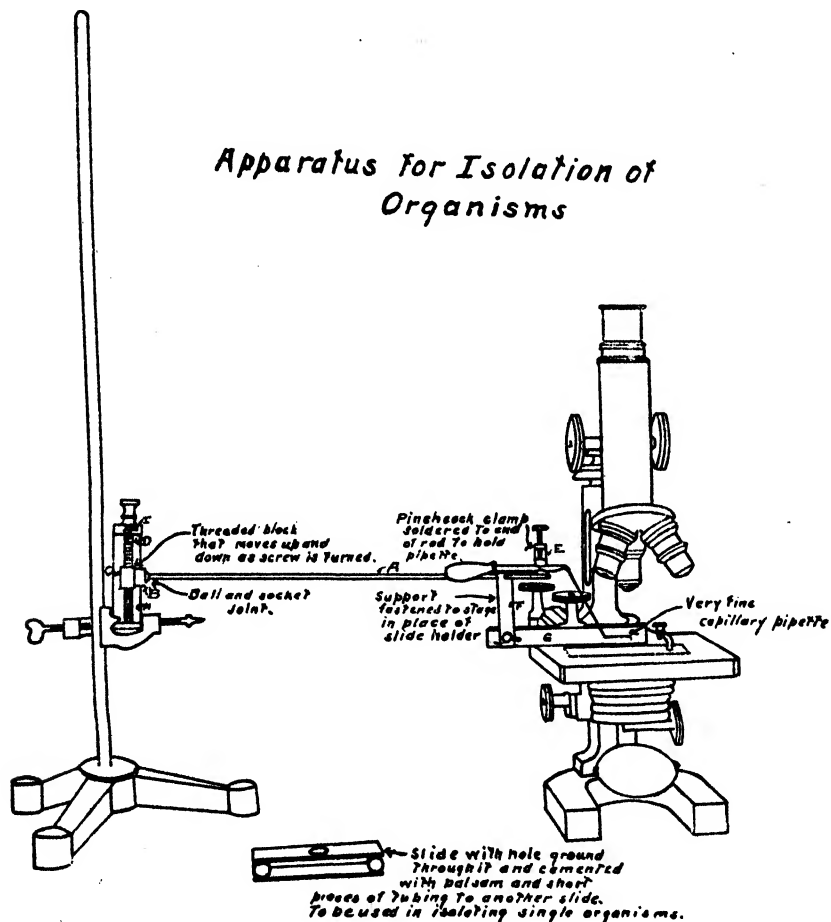


FIG. 1

through holes in each arm of the U. The screw, *D*, has a groove cut around it where it passes through the upper arm of the U into which a small screw, *I*, in the arm fits, preventing the screw, *D*, from moving vertically but allowing it to be turned. The

U-shaped brass, *H*, is fastened to a ring stand by means of an ordinary clamp. By turning the screw, *D*, to the right or left, an up or down movement is imparted to the end of the pipette. The location of the end of the pipette may be adjusted by the rack and pinion movements of the mechanical stage. From this brief description and the illustration, the mechanism of the device may be understood.

By making the threads on screw, *D*, finer or by lengthening the rod, *A*, a finer vertical movement is obtained. The rack and pinions of the mechanical stage allow sufficiently fine adjustment for bacterial work. In order that the movements may be accurate, the threads in block, *C*, should fit the threads of screw, *D*, closely, the ball and socket joint should be carefully made and the bearing in the top of the standard, *F*, should be accurately fitted.

It is not necessary to describe the use of the apparatus since Barber and Chambers have given rather complete details as to the methods to be followed in the micropipette method of isolation.

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A TEST FOR INDOL BASED ON THE OXALIC ACID REACTION OF GNEZDA¹

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There are many methods of testing for indol, but those available for bacteriologic studies are comparatively few.

Baeyer's nitrosoindol reaction of 1870 was introduced into bacteriology by Kitasato in 1889 as a means of differentiating *Bact. coli* from *Bact. typhosum*. The method of application was that developed by Salkowski. It is still recommended in practically all text books, and is widely used in testing for indol production. The Ehrlich para-dimethylaminobenzaldehyde test for use with urine, published in 1901, was applied to bacteriology by Haenen in 1905, and by Böhme in 1906. It has become the test most frequently used for accurate work, either in its original form or with modifications such as that of Steensma.

Gnezda described a pink or purple color reaction formed by the union of oxalic acid and indol in 1899. It is not clear whether Morelli or Pittaluga first applied this reaction to bacteriologic studies. It would appear that they developed the method independently although it was some nine years after the publication of Gnezda's report. Morelli does not refer to Gnezda's work and Zipfel thought that this was simply a return to the principle of the Crisafulli pine splinter hydrochloric acid procedure. The conditions for the two tests are however quite different. Pittaluga calls the test the indol oxalic acid reaction of Gnezda. Four years before these applications to bacteriology Verschaffelt used the method to demonstrate indol from jas-

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mine and orange blossoms. There are only scattered references to the method in bacteriologic literature and most of the current text books have no mention of it. Konrich is reported to have found it unsatisfactory. Zipfel, Baudet and Freund obtained results which compared favorably with other standard methods. It would appear that the method has been either overlooked or underestimated.

One of us (Holman) has used this method since 1911, and has found it very satisfactory. It was controlled by the Ehrlich-Böhme and Salkowski tests, and the results were the same in many hundreds of tests, so that it became the custom to use this test in all cases, and if it failed to show the pink color indicative of indol, to test the medium by the Ehrlich-Böhme method. There were no exceptions to the rule that both tests were negative for the same cultures.

It is generally recognized that there are so many factors in the production of indol by bacteria that the greatest precautions are necessary against drawing wrong conclusions. The results given with the Salkowski and Ehrlich tests frequently fail to agree. It would appear from the studies of Frieber and many previous workers that the Salkowski test is not reliable as a test for indol, since it gives a reaction quite similar when indol acetic acid is formed from the tryptophane molecule. The Ehrlich-Böhme test has been found to react with other compounds than indol (Zoller et al.). The amyl alcohol used for extracting the rosindol must be tested out, as many lots give a color reaction easily confused with indol (Porcher, Telle and Hüber, Baudet). Herter found that indol acetic acid reacted with para-dimethylaminobenzaldehyde, and gave a color similar to that with indol. Frieber did not find this, but rather believed that the Salkowski test is really a test for indol acetic acid. In fact, these tests have been modified and remodified in order to overcome the chances of error, and for careful work the majority of investigators rely on testing the distillates.

The more common method of distilling for indol is the complicated one by means of steam. Zoller has found direct distillation far more simple, and equally satisfactory if certain

precautions are taken. Goré has recently devised an even more direct method of taking advantage of the volatility of indol. The cotton plug of the test tube culture is dipped in the Ehrlich-Böhme solutions, pushed close to the surface of the medium, and the tube gently heated in boiling water. The volatile indol gives the characteristic color to the cotton plug. The oxalic acid test we are considering depends on the volatility of indol at 37°C. or even at room temperature.

In the unsettled state of our knowledge of these color reactions it is advisable where possible to rule out the effect of colored media such as broth, peptone water, etc., if the reagents are to be added directly to the culture media. Zipfel and others have done this in their tryptophane solutions, but we have reason to believe that such poorly buffered solutions are not adapted to the growth of all types of bacteria. The work of Frieber indicated that most of the errors in reading indol reactions are not due to other ingredients in the medium (he used Zipfel's medium modified in various ways) but to the formation by certain bacteria of indol acetic acid and possibly other compounds from tryptophane and related substances when indol is not completely liberated. Without going into detail at this time, it is enough to say that indol acetic acid is not volatile, is not found in the distillates, and will not therefore give the pink color to oxalic acid papers.

The fact that we can narrow our investigations by this simple means to the volatile compounds produced, makes this method important. The rate at which indol volatilizes is rather slow, and the concentration necessary to give the pink color to the oxalic acid paper is not known. In order to determine the dilution of indol which will give the test, 1 mgm. of pure indol was added to 1000 cc. of distilled water. Further dilutions from this stock solution were made, using as the diluting fluid Dunham's solution (Difco peptone) as suggested by Malone and Goré. A dilution containing 0.0009 mgm. per cubic centimeter could be detected after twenty-four hours in the incubator as a slight pink on the oxalic acid paper. Malone and Goré

gave 0.0025 mgm. per cubic centimeter for the ordinary Ehrlich method, 0.0005 for the Goré cotton-wool plug test, 0.0003 for Steensma's test.

The indol content in milligrams per cubic centimeter of broth as estimated by Malone and Goré after twenty-four hours, showed a continued decrease up to seven and fourteen days, undoubtedly due to the volatilizing of the indol. We have been able to test every day, by the oxalic acid papers, the indol volatilizing from cultures up to many weeks in the incubator, and have also shown that the indol is given off even after the cultures have been sterilized by disinfectants such as mercuric bichloride and chloroform. Formaldehyde stopped the reaction. Whether or not a non-volatile compound is here formed is not known. It is of no great practical importance to be able to detect indol in such short times as six hours as Rivas was able to do in his trypsinized pepton media, but it is useful to have a self recording test such as this one which gives the reading for indol when the amount reaches a certain relatively low concentration. We never had a positive test by this method when we used unseeded Dunham's tubes made up with Armour, Difco, Fairchild, Parke and Davis, Will or Witte pepton.

Zoller in a study of the influence of hydrogen ion concentration upon the volatility of indol from aqueous solution shows that the most rapid volatilization of indol takes place over a decidedly alkaline range (pH 8.0 to 10.5) which is of course of direct application to the method we are discussing. Porcher and Panisset emphasized the importance of alkalinizing the medium before distilling. As the deaminizing process in the culture continues with resulting increased alkalinity, the color change of the oxalic acid paper from the volatilizing indol becomes more rapid and distinct. The papers can readily be replaced as desired and the time of maximum volatility, but not necessarily of production, may be thus simply determined.

An interesting and as yet a not readily explainable phenomenon is the fading of the pink color on the paper after several days, which, starting at the extreme lower edge, very slowly decolorizes the whole of the exposed paper. There is some evidence that

it is due to ammonia, and tests with acid fuchsin papers give the maximum ammonia reaction at the edge of this test paper at the same time that the edge of the pink oxalic acid paper is beginning to fade. This phenomenon offers no difficulty in obtaining records, since it takes many days to decolorize the length of the paper, and even after weeks, the paper, where it is held between the glass and the cotton plug, still shows pink.

This volatility of indol can be demonstrated on filter papers, strips of white tape, or even on the absorbent cotton plugs. These are dipped in a saturated watery solution of oxalic acid and allowed to dry. The filter paper should be folded four or five times to prevent it from lying against the side of the tube and to offer a greater surface to the rising indol. The tape may curve under the cotton plug, both ends being held in place. The absorbent plugs can be lightly dipped in the saturated solution and dried in situ, care being taken not to have an excess of crystals on the cotton. It is important to remember that the reaction does not occur if the papers are wet. We have taken untreated absorbent cotton plugs from cultures of indol producing bacteria, and having shaken them in ether, have obtained from the ethereal solution a clear cut, sharp reaction for indol by the Steensma modification of the Ehrlich test. We have also taken the pink oxalic acid papers and treated them in the same way, the solution giving a sharply positive Steensma test.

It would appear that the crystals of oxalic acid must be very small, or distributed on some finely divided material. There was no color change of the oxalic acid crystals deposited on the walls of a Kjeldahl distilling tube where the volatilizing indol must have come in direct contact with the crystals, nor when the indol was allowed to pass up through packed crystals of oxalic acid in a Fresenius filter tube.

The very great advantage of being able to use this method with solid media or colored or various complex media, needs to be emphasized. We have had no trouble in demonstrating indol by a sharp pink reaction on the oxalic acid papers and oxalic acid tape above a culture of *H. influenzae* grown on "chocolate"

(heated blood) agar. A great variety of indol producers grown on agar slants also gave sharp reactions. There is no evidence that this test for indol is less accurate than any other, and it undoubtedly eliminates a great many sources of error liable to occur with the other more commonly used tests.

The advantages of this method of demonstrating indol are many, and they may be briefly restated:

1. It depends on the volatility of indol, with no special methods of distillation. This eliminates a great many substances formed or present in the media which may give confusing color reactions where the tests are made directly in the media.

2. It leaves the tube or flask free for any other test thought necessary or desirable, so that the method is readily compared with other tests.

3. It tells the time of maximum or at least active indol concentration in the simplest way, and thereby eliminates the errors where the test is made too early or too late. The latter possibility is, however, not important, since the indol does not under ordinary conditions disappear from the media for a long time.

4. It can be used with solid media such as plain agar, heated or unheated blood agar, as well as any colored or opaque fluid media which cannot be used with the other common testing methods.

5. It eliminates the chief source of disagreement between the Baeyer-Salkowski nitroso-indol reaction and the Böhme-Ehrlich rosindol test because the compound responsible for this disagreement, indol acetic acid, is not volatile.

6. In a word, it is simple, accurate and practical.

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THE GROWTH OF YEASTS ON SYNTHETIC AGAR MEDIA

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In the course of a study by one of the authors of yeasts found in cream and butter, a medium has been used for plating known as "whey agar." The whey was obtained from skimmed milk by coagulation with rennet. To each 100 cc. of the whey there were added 1.5 cc. of agar and 0.50 grams of pepton. The medium was filtered through absorbent cotton, tubed in 10 cc. portions, and sterilized for twenty minutes at 15 pounds pressure. One cubic centimeter of a 1 per cent tartaric acid solution was added to the Petri dish to keep down bacterial growth.

The successful use of synthetic liquid media for the growth of yeast by Fulmer, Nelson and Sherwood (1921) and Nelson Fulmer and Cessna (1921) led the authors to try to develop a satisfactory synthetic agar medium for the growth of yeast. Such a medium would offer many advantages, chief among them being the possibility of preparing media of more constant composition and properties than would be possible by the use of such variable materials as whey and pepton. These media would serve as basal in a quantitative study of the effect of various chemical environments upon different varieties of yeasts and so might lead to their differentiation and isolation.

EXPERIMENTAL

Three types of organisms were used: *Sacch. cerevisiae*, *T. sphaerica* a lactose fermenting yeast (Hammer and Cordes, 1920); and *Mycoderma*. Tests of the agar media were made both on plates and on slopes. The compositions of the basal media are given in table 1.

The beer wort was made by mashing, at 55°, 360 grams of ground distiller's malt with 1150 cc. of distilled water for twenty-four hours. The mixture was filtered through towelling, then through filter paper and heated for thirty minutes under 15 pounds pressure. After standing for three days to permit coagulated matter to settle out, the liquid was filtered and the agar added. The material was heated in the autoclave at 15 pounds for thirty minutes, filtered through absorbent cotton and tubed. The tubes were sterilized in live steam for thirty minutes on two successive days. The agar was washed several times in distilled water.

TABLE 1

CONSTITUENT	GRAMS PER 100 CC.				
	Medium I	Medium II	Medium III	Medium IV	Medium V
Ammonium chloride.....	0.188	0.188	0.188		
Dipotassium phosphate.....	0.100	0.100	0.100		
Calcium chloride.....	0.100		0.100		
Cane sugar.....	5.00	5.00			
Agar.....	1.50	1.50	1.50	1.50	1.50
Whey.....				Basal	
Beer wort.....					Basal

EFFECT OF VARIATION OF CONCENTRATION OF AMMONIUM CHLORIDE

Fulmer, Nelson and Sherwood (1921) found that optimum reproduction of yeast took place at 30° at a concentration of 0.188 gram of ammonium chloride per 100 cc. of medium. The optimum concentrations of salts in the presence of agar would not necessarily be the same as those for the liquid media.

With medium I as basal, the concentration of ammonium chloride was varied. The colonies were first counted after a period of incubation of one week at 20°C. Table 2 gives a summary of typical experiments.

The actual number of colonies is independent of the concentration of ammonium chloride and this number is practically

identical with that obtained on the whey or wort agar; the synthetic agar media give the same quantitative information as do the usual media used, whey agar and wort agar.

The colonies in the synthetic media are about $\frac{1}{2}$ to $\frac{3}{4}$ the diameter of those obtained on wort or whey agar, *Mycoderma* forming the largest colonies, *T. sphaerica* the smallest, with those of *Sacch. cerevisiae* intermediate. However, the colonies were of sufficient size to make counting very easy. The variation of the concentration of the ammonium chloride did not alter the diam-

TABLE 2

CONCENTRATION OF AMMONIUM CHLORIDE	NUMBER OF COLONIES		
	<i>Sacch. cerevisiae</i>	<i>T. sphaerica</i>	<i>Mycoderma</i>
0.062	41	137	46
0.093	37	151	40
0.124	33	149	32
0.155	34	149	37
0.188	34	136	35
0.217	39	151	37
0.248	33	152	45
Whey agar	38	135	40
Wort agar	36	140	36

eter of the colonies to any appreciable extent. A significant increase in the number of cells would have little effect upon the diameter of the colony. The failure to obtain significant differences in the diameters of the colonies does not in any way contradict previous statements regarding an optimum concentration of ammonium chloride for the reproduction of yeast.

A STUDY OF MEDIA I, II AND III

The three types of yeast grew well on media I and II but showed starved growth on III. *Mycoderma* showed the poorest growth of the three types on Medium III.

In no case, even where there was starved growth, was there a decrease in the number of colonies as compared with those produced on whey or wort agar. The yeasts were able to produce the standard number of colonies on the sugar free medium.

The energy and carbon were derived from the stored carbohydrate material or from the agar. A detailed study is being made of the stored carbohydrate material of these three types.

The colony shapes were typical of the three types on the synthetic media, these forms differing from those typical on whey agar. Table 3 illustrates the differences.

TABLE 3

TYPE	DESCRIPTION OF COLONIES
<i>Saccharomyces cerevisiae</i>	Circular, filamentous. The filaments are composed of long cells with branches at the internodes. On whey or wort agar the colonies are circular with sharp edges.
<i>Mycoderma</i>	Small, irregular, compact growth with characteristic filaments composed of long cells with rosettes of single cells at the internodes. On whey or wort agar the colonies grow as a dull film, round, showing radial formation
<i>T. sphaerica</i>	Small, circular, sharp edged, compact. On whey or wort agar the colonies are circular, convex, smooth, opaque, with entire edge

SUMMARY.

Synthetic agar media may be used for quantitative work with the three types of yeast studied.

Typical colony formation is shown by the three types of yeasts used.

The synthetic agar media furnish poor conditions for the growth of bacteria and are particularly suitable for the growth of yeasts.

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